MECHANISMS FOR GENETICALLY PREDETERMINED DIFFERENTIAL QUANTITATIVE EXPRESSION OF HLA-A AND -B ANTIGENS

By

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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Earlier studies have shown that different specific HLA-A and -B antigens are differentially expressed in cells. Their relative quantities are genetically predetermined and inherited according to Mendelian law. To investigate the mechanisms responsible for the quantitative differential expression of HLA antigens, a simple and reliable method using RT-PCR and DGGE was developed to measure the relative quantities of HLA-A and -B mRNAs in cells. When the relative quantities of different HLA-A and -B proteins expressed in ten different HLA-phenotyped lymphoblastoid cell lines (LCLs) were correlated with the relative amounts of their respective mRNAs in cytoplasm, it was shown that HLA protein levels are proportional to their mRNA levels except for the cell lines that were positive for HLA-A24 and -B7. This finding suggests that different protein translational efficiencies could play some role in affecting differential expression of HLA antigens. An in vitro translation study using HLA-A24 and -B60 mRNAs supports the hypothesis that HLA-A24 mRNAs are more efficient in synthesizing HLA heavy chains. To determine the stability of different HLA-A and -B antigens expressed in HLA-phenotyped LCLs, it was found that different HLA-A and -B antigens have

similar turnover rates. Measurement of the relative quantities of HLA-A and -B mRNAs in seven LCLs before and after treatment with DRB, an inhibitor of RNA polymerase II, showed that different specific HLA-A and -B mRNAs in five LCLs have the same turnover rates and that HLA-A and -B mRNAs are not proportionally degraded in the other two LCLs. Measurement of the relative quantities of different HLA-A and -B pre-mRNAs in nuclei showed that they are not proportional to those of mature cytoplasmic mRNAs in five of seven HLA-phenotyped LCLs. All these findings indicate that the quantitative differential expression of HLA-A and -B antigens is regulated by a combination of multiple steps. These steps include gene transcription, pre-mRNA splicing, mRNA degradation and/or mRNA translation, depending on specific HLA alleles in different individuals. Despite the complexity of regulating the differential quantitative expression of HLA antigens, all the aforementioned mechanisms are encoded in the sequences of HLA gene. These findings support the earlier observations that relative quantities of different HLA-A and -B antigens are genetically predetermined, and directly linked to HLA-A and -B genes and inherited according to Mendelian laws.

CHAPTER 1
INTRODUCTION

Biochemistry of Class I HLA Molecules

Class I HLA molecules are polymorphic membrane glycoproteins and consist of two noncovalently associated polypeptide chains -- a heavy chain of 44 kD encoded by the classical class I HLA genes (HLA-A, -B, and -C) and an invariant light chain of 12 kD encoded by a non-MHC gene, β2 microglobulin (β2m) (Srivastava et al., 1985). The HLA heavy chain is a type II transmembrane protein comprising a cytoplasmic carboxyl terminal domain, a transmembrane segment, and three extracellular domains known as α1, α2 and α3. The α1 and the α2 domains contain most polymorphic amino acid sequences and form a binding groove for antigenic peptides. This peptide-binding groove consists of an eight-stranded antiparallel β-sheet flanked by two parallel strands of α-helices (Bjorkman and Parham, 1990). The binding groove can accommodate antigenic peptides of eight to ten amino acids in a flexible, extended conformation (Falk et al., 1991). However, an earlier study also showed that peptides consisting of twelve amino acids can bind to class I HLA molecules (Bednarek et al., 1991).

Organization Of Class I HLA Genes

Genes for class I HLA heavy chains are located on the short arm of chromosome 6, and the gene for β2m is on chromosome 15. There are three loci for classical class I HLA genes. All three functional HLA class I genes share a very similar genomic

organization and are responsible for encoding HLA-A, -B, and -C heavy chains. Each class I HLA gene consists of eight exons, and the exon-intron organization reflects the domain structure of the molecule (Figure 1). The exon 1 encodes the 5'-untranslated region (5'-UTR) and the signal sequence, and the exons 2, 3 and 4 encode the α1, α2, and α3 (immunoglobulin-like extracellular region) domains of HLA heavy chains, respectively. The transmembrane region (TM) is encoded by exon 5, and the cytoplasmic tail (CY1 and CY2) and the 3'-untranslated region (3'-UTR) are encoded by exons 6-8 (Srivastava et al., 1985; Ways et al., 1985). There are only minor differences among HLA-A, -B and -C genes at these three different loci. HLA-B genes, unlike HLA-A and -C genes, do not have coding sequences in exon 8. HLA-C genes contain extra three nucleotides in exon 5 (Davidson et al., 1985; Strachan et al., 1984). These three nucleotides are not present in HLA-A and -B genes. In addition to these three functional classical HLA class I genes, there are also three nonclassical HLA class I genes, HLA-E, -F and -G, and several pseudogenes (Le Bouteiller, 1994).

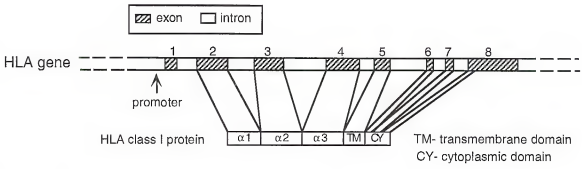


Figure 1 General organization for class I HLA genes

Polymorphism and Phenotypes of Class I HLA Antigens

One of the important features of class I HLA gene products is a high degree of polymorphism. At present, there are at least 86 alleles for HLA-A locus, 186 alleles for

HLA-B locus, and 46 alleles for HLA-C locus (Bodmer et al., 1997; Mason and Parham, 1998). The structural basis of allelic polymorphism has been well characterized by direct comparison of protein and nucleotide sequences (Parham et al., 1995; Parham et al., 1988). The observed protein polymorphism is due to amino acid substitutions. Most polymorphic amino acid residues are confined to the peptide-binding region in $\alpha 1$ and $\alpha 2$ domains and contribute to varying peptide-binding specificities of class I HLA molecules (Parham et al., 1988). The HLA polymorphism is therefore responsible for presenting large numbers of diverse antigenic peptides restricted to specific HLA molecules (Bjorkman and Parham, 1990). Despite significant degrees of variations in nucleotide and amino acid sequences, HLA alleles at the same locus are evolutionally more closely related to one another than HLA alleles at other loci.

Traditionally, phenotypes of class I HLA antigens are determined serologically. This approach is based on complement-mediated lymphocytotoxicity and was originally developed by Terasaki and McClelland (Terasaki et al., 1978). In this assay, peripheral blood lymphocytes are incubated with an antiserum containing specific anti-HLA antibodies. The binding of antibodies to lymphocytes is then detected by adding heterologous complement. Subsequent cell death induced by the activated complement is scored after staining with eosin-Y or other vital dyes. Although the microlymphocytotoxicity assay for typing HLA antigens is sensitive and convenient, this assay suffers from cross-reactivity and limited specificity of typing sera.

Another technique that has been used to identify the phenotypes of class I HLA antigens is isoelectric focusing (IEF) gel electrophoresis (Yang, 1989). In this method, the HLA class I antigens are digested with neuraminidase and separated on IEF gels based on their isoelectric points. The separated HLA heavy chains are then detected by class I HLA specific antibody. This technique is able to resolve the subtypes of various HLA antigens that can not be differentiated by the lymphocytotoxicity assay. However, some HLA

subtypes remain unresolvable by IEF gel electrophoresis. The IEF approach is also technically cumbersome.

More recently, molecular biology techniques have been applied to DNA sequence-based HLA typing (Allen et al., 1994; Bidwell, 1994; Gao et al., 1994; Oh et al., 1993; Tiercy et al., 1994). Three commonly used approaches are: (1)locus-specific PCR followed by hybridization with sequence-specific oligonucleotides; (2) one-step PCR with sequence-specific primers; (3) locus-specific PCR followed by DNA sequencing (Bidwell, 1994). These typing methods provide the most accurate results. However, these new techniques are laborious.

Function of Class I HLA Antigens

Functionally, class I HLA antigens play important roles in the host immune system. They are expressed on almost all cells including anucleated red blood cells and platelets (de Villartay et al., 1985; Everett et al., 1987; Mueller-Eckhardt et al., 1985; Rivera and Scornik, 1986). After being synthesized, HLA heavy chain and β2m form heterodimers in the endoplasmic reticulum (ER), where they are loaded with peptides generated from cytosol by the proteasome. The proteasome is a multisubunit ATP-dependent protease that plays the major role in normal turnover of cytosolic proteins (Pamer and Cresswell, 1998; Tanaka et al., 1997). The peptides generated by the proteasome are translocated into the ER by the transporter associated with antigen processing (TAP), which is a heterodimeric protein that belongs to the ATP-binding cassette transporter family (Momburg and Hammerling, 1998). Before binding with the antigenic peptide, a transient complex containing a class I heavy chain and a β2m is assembled onto the TAP molecule by successive interactions with the ER chaperones calnexin, calreticulin and tapasin. The current model suggests that, before binding antigenic peptides, newly synthesized HLA heavy chains first bind calnexin. After β2m binds, calnexin is exchanged for calreticulin.

Then tapsin mediates the association of the HLA class I heavy chain-β2m-calreticulin complex with TAP which provides the peptides for the assembly of class I HLA antigen. After binding of the antigenic peptide, the HLA molecules are transported to the cell surface (Parner and Cresswell, 1998). The misfolded class I heavy chains without β2m-association or peptide-binding are translocated to the cytoplasm and degraded by the proteasome (Hughes et al., 1997).

The antigen peptides presented by class I HLA molecules include peptides of host and non-host cellular proteins. The latter include the proteins derived from invading virus, bacterium, or protozoan parasite. The presentation of pathogen-encoded or host tumor antigenic peptides by class I HLA molecules on cells plays a crucial role in immune elimination of tumor cells or pathogen-infected cells by $CD8^+$ T cells (Bjorkman et al., 1987; Monaco, 1992). The presentation of peptides by class I MHC molecules in the thymus also plays a crucial role in the selection and maturation of $CD8^+$ T cells. During this process, the $CD8^+$ T cells bearing the T cell receptors with high affinity to self antigens are negatively selected, whereas those with lower affinity to self antigens in the context of self MHC molecules are positively selected to mature and leave the thymus to populate peripheral lymphoid tissues (Robey and Fowlkes, 1994).

In addition, the expression of class I HLA molecules on cells has been implicated in protecting host cells from destruction by autologous natural killer cells (Ciccone et al., 1994; Kaufman et al., 1993; Litwin et al., 1993). The existence of inhibitory NK cell receptors for polymorphic classical HLA class I molecules prevents the attack of normal host cells by NK cells and could be responsible for elimination of the cells lacking sufficient expression of HLA class I molecules (Lanier, 1998). It has also been shown that peptides derived from signal sequences of HLA class I heavy chains can be presented to NK cells by a non-classical HLA class I molecule, HLA-E (Braud et al., 1998).

Due to the highly polymorphic nature of class I HLA antigens, they are responsible for the immune rejection of allografts in transplant recipients (Hood et al., 1983).

Although HLA matching between recipient and donor does not in itself completely prevent rejection of allografts, it significantly improves the clinical outcome for allogeneic organ transplantation. Despite the proven success of matching HLA antigens for transplantation, unrelated individuals who are serologically typed as HLA-identical often do not share identical HLA antigens due to limited availability to differentiate various HLA subtypes by serological assays (Mantovani et al., 1995; Speiser et al., 1996). The use of molecular biology techniques for HLA typing at the level of DNA sequence will further prevent mismatches and may improve the results for allograft transplantation.

Association of Class I HLA Antigens with Diseases

Owing to their role in antigen presentation, class I HLA molecules are closely involved in the pathogenesis of various clinical conditions, which include infectious, autoimmune and neoplastic diseases. The association of certain HLA alleles with diseases has also been documented (Hall and Bowness, 1996; Hill, 1998). One of the strongest associations is the association of HLA-B27 with ankylosing spondylitis. It has been shown that HLA-B2701, 02, 04 and 05 are associated with the development of ankylosing spondylitis (Breur-Vriesendorp et al., 1987; D'Amato et al., 1995; Hill et al., 1991a; Lopez-Larrea et al., 1995). Associations of HLA-B51 with Behcet's disease, HLA-B52 and HLA-B3902 with Takayasu's arteritis, HLA-A2902 with birdshot retinitis, and HLA-B27 with reactive arthritis have also been reported (Brewerton et al., 1974; Laitinen et al., 1977; LeHoang et al., 1992; Mizuki et al., 1993; Mizuki et al., 1992; Toivanen et al., 1994; Yoshida et al., 1993). The underlying pathogenic mechanisms for these associations are still unclear. It is likely that their roles may be associated with peptide binding specificity by different HLA molecules and/or by thymic selection of specific T cell repertoire responsible for the diseases (Hall and Bowness, 1996).

For infectious diseases, HLA-B53 has been shown to be associated with the protection from severe malaria (Hill et al., 1991b). It is likely that HLA-B53 molecules may confer the resistance by presenting a plasmodium-derived peptide to CTL for mounting an effective immune response (Hill et al., 1991a; Hill et al., 1992). Undoubtedly, further understanding of the functional importance of HLA antigens in determining host defense and disease susceptibility will be made in the near future when more information becomes available.

Controlling Steps in Gene Expression

The mechanism for gene expression has been extensively studied. Gene expression can be controlled at multiple steps, including gene transcription, pre-mRNA processing, mRNA trafficking, mRNA degradation, mRNA translation, and protein turnover. It is generally believed that gene transcription is the most critical step in controlling gene expression. Gene transcription can be regulated by many cis-acting elements and trans-acting factors (Olave et al., 1997; Yanofsky, 1992). The TATA box in the promoters of various genes is crucial because it serves as a common recognition site for transcription factor TFIID and for the assembly of the RNA polymerase II initiation complex. There are also enhancers and silencers that bind various trans-regulating factors. To initiate transcription of a gene, at least six different transcription factors together with RNA polymerase II are required to form a transcriptionally competent preinitiation complex (Olave et al., 1997; Yanofsky, 1992). Regulation of transcription therefore can be accomplished by controlling assembly of the preinitiation complex or the catalytic efficiency of RNA polymerase II during initiation, elongation, or termination (Hampsey, 1998). The detailed mechanisms for transcription and its regulation remain to be elucidated.

Processing of pre-mRNA by capping, splicing, editing and polyadenylation is also important in regulating availability of mature mRNA for protein synthesis. For pre-mRNA

splicing, the assembly of a spliceosome, consisting of a pre-mRNA, protein factors and small nuclear ribonucleoproteins (snRNPs), is required (Moore and Sharp, 1993). Thus, the splicing of pre-mRNA is regulated through assembly of the spliceosome. In some cases, pre-mRNAs can be spliced in alternative ways leading to the production of different isoforms of proteins (Hodges and Bernstein, 1994). It appears that alternative splicing in many systems is controlled through regulation of the amount, the distribution, and/or the activity of constitutive splicing factors in cells (Bernstein and Hodges, 1997). It has also been demonstrated that alternative splicing can be affected by differences in the strength of competing 3' and 5' splice sites, large distances between polypyrimidine tracts and 3' splice sites, size of the involved exon, steric constraints on splicing factor binding, and alternative polyadenylation sites (Balvay et al., 1993; Brady and Wold, 1987; Carstens et al., 1998; Elrick et al., 1998; Eperon et al., 1988; Furdon and Kole, 1988; Heinrichs et al., 1998; Jin et al., 1998; Lim and Sharp, 1998; Nelson and Green, 1990; Norbury and Fried, 1987; Peterson et al., 1994; Solnick, 1985; Solnick and Lee, 1987; Sterner and Berget, 1993; Watakabe et al., 1989).

Gene expression is also regulated by degradation and stability of mRNA. After export of mRNA from the nucleus to the cytoplasm, the stability and turnover of mRNA can contribute significantly to the control of gene expression through regulation of the available mRNA for protein synthesis (Ross, 1995). Different mRNAs can have different intrinsic rates of turnover (Cabrera et al., 1984; Carneiro and Schibler, 1984). Many examples for regulation of gene expression by modulation of mRNA decay have been documented (Caponigro and Parker, 1996; Ross, 1995). Stability of mRNA can regulated by cis-acting elements within the mRNA molecule. These cis-acting elements serve as recognition sites for various regulatory proteins including poly(A)-binding protein (PABP), AU-rich element (AURE)-binding proteins (AUBPs) and other proteins that bind to the 3'-UTRs or coding regions of mRNAs (Beelman and Parker, 1995; Ross, 1995; Ross, 1996; Sachs, 1993). The aforementioned cis-elements can positively or negatively modulate

mRNA stability, and are present throughout the mRNA molecule in 5'-UTR, the coding region and 3'-UTR (Tharun and Parker, 1997).

Translation of mRNA is another step in regulating gene expression, and it can be regulated by modulating the rate of translational initiation and/or sequestering mRNAs in translationally inaccessible messenger ribonucleoprotein (mRNP) (Curtis et al., 1995). The concentration of active initiation factors and the primary sequence of the 5'-UTR have been shown to influence the rate of mRNA translation (Devarajan et al., 1992; Hess and Duncan, 1994; Kanaji et al., 1998; Lincoln et al., 1998; Thach, 1992). The sequence flanking the translation initiator AUG, 5' cap, the secondary structure in the 5'-UTR, the presence of alternative translation initiation sites and the length of 5'-UTR have been shown to determine the intrinsic translational efficiency of mRNAs (Bhasker et al., 1993; Falcone and Andrews, 1991; Gallie, 1991; Gallie and Tanguay, 1994; Gambacurta et al., 1993; Gray and Hentze, 1994; Iizuka et al., 1994; Ito et al., 1990; Lawson et al., 1986; Lopez-Casillas and Kim, 1991; Pinto et al., 1992; Rao and Howells, 1993; Sedman et al., 1990; Yun et al., 1996). The poly(A) tail at the 3' ends of eukaryotic mRNAs also can serve as an enhancer for mRNA translation (Jacobson, 1996).

Therefore, it is of interest to learn how these different controlling steps are involved in regulating differential quantitative expression of various specific HLA-A and -B antigens in cells.

Regulation of Quantitative Class I HLA Gene Expression

The expression of class I HLA genes in cells is regulated both positively and negatively by the interaction of different trans-acting factors with cis-regulatory elements at the genomic level as mentioned earlier. This subject has been reviewed in detail previously (David-Watine et al., 1990). The molecular mechanisms for regulating the expression of class I MHC genes has only been partially elucidated. The transcription of class I HLA

genes is controlled by different regulatory elements in the 5'-flanking region. The cis-regulatory elements that have been identified include promoter sequences (TATA box, CCAAT box), the class I regulatory elements (CRE/enhancer A), κB enhancer elements, an interferon response sequence (IRS), the negative regulatory element (NRE), and the R x Rβ binding motif. Various trans-acting factors for these elements have also been described (Blanar et al., 1989; Driggers et al., 1990; Kieran et al., 1990; Waring et al., 1995; Yano et al., 1987).

As mentioned earlier, alternative splicing of pre-mRNAs may regulate gene expression by diverting some of the gene transcripts into synthesizing different forms of proteins. It has been shown that class I HLA antigens are present in water-soluble form in plasma (Charlton and Zmijewski, 1970; Kao, 1987; Krangel, 1987; Rood et al., 1970). Further biochemical analysis demonstrated that the 39-kD water-soluble form of class I HLA heavy chain is the translation product of an alternatively spliced HLA mRNA without exon 5 (Haga et al., 1991). Because exon 5 encodes the transmembrane domain, the protein product of the mRNA without transmembrane domain is secreted into extracellular fluids. The presence of high concentrations of water-soluble form of HLA antigens has been associated with HLA-A24 phenotype (Adamashvili et al., 1996; Kao et al., 1988; Krangel, 1987). At present, it is not known how alternative splicing could affect HLA expression on cells. Because the alternatively spliced HLA transcripts are only present in low quantities in cells (Haga et al., 1991), it is unlikely that alternative splicing plays a significant role in regulating quantitative HLA expression.

The assembly and transportation of the class I HLA-β2m-peptide complex also can affect the expression of class I HLA antigens on the cell surface. It has been shown that class I HLA antigens are absent on the cell surface of β2m-deficient cells, and that the expression of class I HLA antigens can be restored after the cells are transfected with β2m (Ljunggren et al., 1990; Powis et al., 1991; Tarleton et al., 1992). Because the antigenic peptides are essential for the assembly of class I HLA antigens, both generation of

antigenic peptides in the cytoplasm and transportation of these peptides into ER are critical for the expression of class I HLA antigens. Inhibition of proteasomes can result in reduced availability of binding peptides and lead to decreased expression of class I MHC antigens on the cell surface (Benham and Neefjes, 1997; Grant et al., 1995). Mutation of TAPs can decrease the expression of HLA antigens by limiting the supply of antigenic peptides. Transfection of these mutant cells with native forms of TAP cDNAs is able to restore the normal expression of class I HLA antigens (Hughes et al., 1997). However, the availability of β2m or antigenic peptides does not appear to play a significant role in regulating quantitative expression of class I HLA in normal cells. For instances, polymorphism of TAPs does not affect quantitative expression of class I HLA (Daniel et al., 1997), and expression of different HLA-A and -B antigens in cells is proportionally upregulated during viral infection, interferon stimulation or transformation by EBV virus (Shieh and Kao, 1995). These findings suggest that antigenic peptides are present in abundance and readily available in ER for binding by different specific HLA-A and -B antigens. Therefore, the availability of antigenic peptide is not a rate-limiting step in controlling HLA expression in cells with normal functional proteasomes and TAPs. Although a great deal of information has been gained in how HLA expression is regulated in general, it is not known how different HLA-A and -B antigens are proportionally upregulated in cells in response to interferon or infection by certain viruses.

Functional Importance of Quantitative Expression of HLA Antigens

Many recent studies have concentrated on identifying the antigenic peptides that bind to HLA molecules and little attention has been paid to potential functional importance of quantitative expression of HLA antigens. Earlier study (Bukowski and Welsh, 1985a) suggested that the upregulation of HLA expression during influenza virus infection could enhance the susceptibility of infected cells to CTLs. To further investigate the potential

quantitative importance of HLA antigens in determining the susceptibility to CTLs, Shieh and Kao conducted a series experiments and demonstrated a linear quantitative correlation between HLA-A2 antigens expressed on target cells and the susceptibility of these cells to HLA-A2 restricted CTLs (Shieh et al., 1996). These findings support the potential quantitative importance of HLA antigens. The importance of quantitative HLA expression is also supported by the findings that tumor cells or virus-infected cells can escape immune surveillance through down regulation of HLA expression (Bodmer et al., 1993; Honma et al., 1994; Ruiz-Cabello et al., 1991). In contrast, treatment with IFN-γ to restore MHC class I expression enhances the susceptibility of these cells to CTLs (Peltenburg et al., 1993; Versteeg et al., 1988; Versteeg et al., 1989b).

It has been known that during infection by certain viruses, such as adenovirus, herpes simplex virus (HSV), human immunodeficiency virus (HIV) and cytomegalovirus (CMV), the expression of class I HLA antigens is greatly reduced (Anderson et al., 1985; Ehrlich et al., 1989; Gosgusev et al., 1988; Hill et al., 1995; Howcroft et al., 1993; Walev et al., 1992). The reduced expression of HLA antigens may contribute to the successful evasion of viruses from host cellular immune response. All of these findings support the functional importance of quantitative expression of HLA antigens. Both increased and reduced expression of HLA expression can influence the host susceptibility, severity and recovery from various clinical conditions as described above.

Quantitative Differential Expression of HLA-A and -B antigens

The regulation of quantitative expression of class I HLA antigens has been widely studied (Bishara et al., 1988; Gerrard et al., 1988; Girdlestone and Milstein, 1988; Hakem et al., 1989; Leeuwenberg et al., 1987; Masucci et al., 1989; Masucci et al., 1987; Ohlen et al., 1989; Shimizu and DeMars, 1989; Versteeg et al., 1989a; Zachow and Orr, 1989). The reported studies suggested that the expression of different HLA-A and -B genes are

differentially expressed and upregulated. However, most of these studies were conducted using tumor cell lines or cells transformed with class I HLA cDNA constructs or genes. For this reason, results obtained from these studies can not be extrapolated to native HLA-A and -B genes in normal cells.

When the quantitative expression of native HLA genes was studied, it was found that the relative quantities of HLA-A and -B antigens in different types of cells of an individual are the same and remain unchanged over time (Kao, 1989; Kao and Riley, 1993). These findings suggest that the relative amounts of different class I HLA antigens expressed on cells are genetically predetermined. Subsequent studies of comparing the quantitative expression of different specific HLA antigens in members of different HLA phenotyped families confirmed that the differential quantitative expression of HLA-A and -B antigens is linked directly to class I HLA genes and follows Mendelian inheritance (Kao and Riley, 1993). In addition, it was found that the relative amounts of different specific HLA antigens are proportionally amplified during up-regulated expression of total class I HLA antigens by interferon treatment, EBV transformation or infection with influenza viruses (Shieh et al., 1996; Shieh and Kao, 1995). Because the amount of HLA antigens expressed on cells has been shown to proportionally affect the susceptibility of cells to cytotoxic T lymphocytes (Shieh et al., 1996), and the quantities of each specific HLA antigen expressed on cells may influence disease susceptibility, severity and recovery as discussed earlier, it is of importance to learn what mechanisms are employed to control the genetically predetermined quantitative differential expression of HLA-A and -B antigens. The goal of my dissertation research is to determine how varied quantitative expression of HLA antigens on cells of an individual is controlled by gene transcription, mRNA turnover, mRNA translation, and/or protein degradation. Specifically, experiments are conducted (1) to determine whether different HLA-A and -B proteins in cells are proportionally degraded; (2) to determine whether the relative quantities of HLA-A and -B antigens expressed in cells are proportionally correlated with the levels of mRNAs for these

antigens; (3) to determine whether mRNAs for different HLA-A and -B antigens in cells have the same stabilities; and (4) to determine whether different HLA-A and -B mRNAs are differentially produced.

CHAPTER 2
MEASUREMENT OF RELATIVE QUANTITIES OF DIFFERENT HLA-A AND -B MRNAS IN CELLS BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION AND DENATURING GRADIENT GEL ELECTROPHORESIS

Introduction

As discussed in Chapter 1, class I HLA antigens are polymorphic membrane glycoproteins that consist of a 44-kD heavy chain and a 12-kD invariant β2-microglobulin (Ploegh et al., 1981). The genes encoding HLA heavy chains are located at three different loci (A, B, C) of chromosome 6 (Ploegh et al., 1981). Functionally, class I HLA antigens play important roles in presenting antigen peptides to $CD8^+$ cytotoxic T cells (Zinkernagel and Doherty, 1979) and are essential for the development of $CD8^+$ CTLs in the thymus (Koller et al., 1990; Zijstra et al., 1990). The quantity of HLA antigens expressed on cells also play an important role in determining the susceptibility of virus-infected cells to CTLs (Bukowski and Welsh, 1985b; Shieh et al., 1996). Moreover, HLA-A and -B antigens are expressed in different quantities in cells according to Mendelian inheritance (Kao and Riley, 1993).

However, the molecular basis for the genetically predetermined differential quantitative expression of class I HLA-A and -B antigens in cells remain unknown. In order to address this question, it is necessary to develop a method for measuring the relative quantities of different HLA-A and -B mRNAs in cells. Although several methods for quantitation of specific mRNAs are available including northern blot, S1 nuclease or ribonuclease protection assay and quantitative reverse transcription-polymerase chain reaction (RT-PCR), none of these methods could be easily applied to our study due to technical complexity and/or problems of cross hybridization resulting from high degrees of

sequence homology for HLA mRNAs. We therefore exploited the simplicity of quantitative RT-PCR and the high resolution power of denaturing gradient gel electrophoresis (DGGE) to develop a simple and reliable method for measurement of the relative quantities of different HLA-A and -B mRNAs in cells. The procedures and the validation of this method are described herein.

Materials and Methods

Lymphoblastoid Cell Lines and RNA preparation

EBV-transformed lymphoblastoid cell lines (LCLs) with well characterized class I HLA phenotypes were obtained from the American Society for Histocompatibility and Immunogenetics Cell Repository (Yang et al., 1989) or developed in our laboratory (Shieh and Kao, 1995). These cell lines were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% fetal calf serum, 1% antibiotic-antimycotic solution and 40 μg/ml gentamycin. Total cytoplasmic RNA was isolated using the RNAeasy Total RNA Kit (QIAGEN Inc., Chatsworth, CA) according to the manufacturer's protocol.

Reverse Transcription of mRNAs

First-strand HLA cDNAs were prepared by reverse transcription of total cytoplasmic RNA in 50 μl reverse transcription buffer containing 1.5 μM HLA-specific primers, 0.5 mM dNTP, 10 μM DTT, 100 U rRNasin (Promega Corp., Madison, WI) and 500 units M-MLV reverse transcriptase (Life Technologies, Grand Island, NY) at 37°C for 2 hours. Thereafter, the reverse transcriptase was inactivated by heating at 99°C for 5 min. The primer (5'-TTG AGA CAG AGA TGG AGA CA-3'), which is complementary to a nucleotide sequence conserved among all class I HLA-A, -B, and -C mRNAs in the 3'-untranslated region (UTR) nucleotide sequence (Davidson et al., 1985), was used to

prepare the first-strand HLA cDNA containing the whole coding region. The synthesized cDNAs were subsequently used as templates for PCR to obtain the whole coding sequences for cloning. Another primer (5'-ACA GCT CC(A,G) (A,G)TG A (C,T)C ACA-3'), which is specific and complementary only to the nucleotide sequences of exon 5 of all class I HLA-A and -B genes, but not to those of HLA-C genes, was used to synthesize the first-strand HLA cDNA. The HLA-A and -B cDNAs then were used for quantitative RT-PCR to determine the relative amounts of different HLA-A and -B mRNAs by DGGE and phosphor imaging analysis. The use of this primer enables us to avoid possible interference by HLA-C mRNAs.

Polymerase Chain Reaction (PCR) and TA cloning

To obtain the whole coding sequences of class I HLA cDNA for cloning, a pair of primers that are specific for all class I HLA mRNAs and encompass the 5'-UTR and the 3'-UTR nucleotide sequences of HLA mRNAs (Ennis et al., 1990) were used for PCR. The sequences of this pair of primers are 5'-GAA TCT CCC CAG ACG CCG AG-3' and 5'-TCA GTC CCT CAC AAG ACA GC-3', respectively. The cDNA templates for PCR were prepared as described in the previous section. The PCR was performed in buffer containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, supplemented with 0.2 mM of each dNTP, 0.5 μM of each primer and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in a volume of 100 μl for 25 cycles. Each cycle consisted of 94°C denaturation for 1 min, 65°C annealing for 1 sec and 72°C extension for 1.5 min. PCR products were cloned into a PCR™II plasmid vector using a TA Cloning Kit (Invitrogen, San Diego). HLA specificities of the plasmids isolated from these TA clones were determined by automated DNA sequencing using Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The primer sequence for DNA sequencing is 5'-GCG ATG TAA TCC TTG CCG-3' and complementary to the nucleotides 429-446 of class I HLA coding sequence.

The nucleotide sequences of the primers used for quantitative PCR are 5'-CGC CGT GGA TAG AGC AGG-3' and 5'-GCG ATG TAA TCC TTG CCG-3', which are complementary to the conserved antisense and sense nucleotide sequences in exon 2 and exon 3 of all class I HLA mRNAs, respectively. Quantitative PCR was performed using the same conditions as described above in a volume of 50 µl except annealing at 60°C for 0.5 min, extending at 72°C for 1 min and amplified for 18 cycles. The HLA-A and -B cDNA prepared from cytoplasmic RNA was used as templates.

^{32}P Labeling of The Primer for Quantitative PCR

Four hundred picomoles of a primer was labeled with 54 pmoles of [γ-^{32}P]ATP (3000 Ci/mmol, 10mCi/ml) (Amersham Life Science, Inc., Arlington Heights, IL) in 80 µl pH 7.6 buffer containing 70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT and 40 units of T4 kinase (Promega, Madison, WI) at 37°C for 1 hour. The free nucleotides were removed by Sephadex G-25 filtration. Specific activity of the labeled primer was about 1x 10^5 cpm/pmole.

DGGE

DGGE was performed in 1 mm thick 6% polyacrylamide (acrylamide: bisacrylamide = 19:1) gel using D-GENE™ Denaturing Gradient Gel Electrophoresis System (Bio-Rad Laboratories, Hercules, CA). The polyacrylamide gel contained a linearly increasing denaturant gradient from 40% to 60%. The 100% denaturant contains 7 M urea and 40% (w/v) deionized formamide. Electrophoresis was performed at 60°C, 165 V for 2 to 3.4 hours in 40 mM Tris-acetate, pH 8.0, containing 1 mM EDTA (TAE). After electrophoresis, the gels were stained with ethidium bromide and photographed by using a Polaroid camera or Gel Print 2000i system (Bio Photonic, Corp., Ann Arbor, MI), or dried for autoradiography and phosphor imaging analysis. PCR product of each HLA-A or

-B mRNA in DGGE gel was identified by using PCR products prepared from the plasmids containing cloned HLA-cDNAs of known specificity.

Preparation of HLA mRNA Standards

HLA-A and -B cDNAs cloned in the PCR™II vector (Invitrogen, San Diego, CA) were used for in vitro synthesis of the RNA standards. Five micrograms of plasmid was digested with 20 units of *Bam*H I in a volume of 100 µl at 37°C for 2 hours. After confirmation of complete digestion by agarose gel electrophoresis, linearized plasmids were isolated by phenol/chloroform extraction and ethanol precipitation. One microgram of linearized plasmids were used as templates for in vitro transcription in a volume of 20 µl at 37°C for 4 hours using T7 RNA polymerase according to the manufacturer's protocol (MEGAscript™ In Vitro Transcription Kits) (Ambion Inc., Austin, TX). After 4 hours incubation, 2 units RNase-free DNase I was added and incubated at 37°C for 30 minutes to degrade the template DNA. The in vitro synthesized RNA transcripts were recovered with phenol/chloroform extraction and isopropanol precipitation. Free nucleotides were removed by RNeasy spin column separation (QIAGEN Inc., Chatsworth, CA) and ethanol precipitation. The concentrations of the synthesized RNA standards were measured by absorbance at 260 nm. These transcripts were used as standards for quantitative RT-PCR and S1 nuclease protection assay.

S1 Nuclease Protection (SNP) Assay for Quantitation of HLA-A and -B mRNAs in Lymphoblastoid Cell Lines

The DNA probes for S1 nuclease protection assays were generated from plasmids in which the coding sequence 218-446 of HLA-A or -B mRNA was cloned. DNA containing this partial HLA-A or -B nucleotide sequence and the flanking polycloning site sequences in the vector was amplified using PCR with a pair of primers flanking the polycloning sites. The PCR products were then purified by agarose electrophoresis and

Sephaglas™ BandPrep Purification Kit (Pharmacia LKB), and used as templates for probe synthesis. The probes were synthesized by using an anti-sense primer that is complementary to the coding sequence between nucleotide 429 and 446 of HLA-A and -B mRNA, a Prime-A-Probe™ DNA labeling Kit (Ambion, Inc., Austin, TX) and labeled with [α-^{32}P]dATP. After gel purification, the DNA probes were used for quantitative S1 nuclease protection assay using S1-Assay Kit (Ambion, Inc., Austin, TX) according to the manufacturer's instruction. HLA-A and -B RNA transcripts synthesized from the cloned cDNAs were used to construct standard curves. For quantitation of HLA mRNAs, 0.5-1 µg of total cytoplasmic RNA was assayed in triplicates. The S1 nuclease-digestion products were separated using a 6% denaturing polyacrylamide gel (8M urea). The protected DNA fragments were quantified by phosphor imaging. The amount of specific HLA-mRNA was determined from the standard curve.

Autoradiography, Phosphor Imaging and Densitometry

For autoradiography, the gels were exposed to Fuji Medical X-ray film (Fuji Photo Film Co., Ltd., Japan) for 3 hours (DGGE) or 48 hours (SNP assay) at -70°C. For phosphor imaging, the gels were exposed to a Phosphorscreen (Molecular Dynamics, Inc., Sunnyvale, CA) for 3 hours (DGGE) or 24 hours (SNP assay) at room temperature. The radioactivity of each specific DNA band was quantified using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). For quantitation of DNA in agarose gels by densitometry, photographs of gels were scanned with a 600 dpi Microtek grayscale scanner (Microtek, Inc., Torrance, CA) and analyzed by Collage 3.0 software (Fotodyne Inc., New Berlin, WI).

Results

Optimization of Quantitative RT-PCR

Although the amount of PCR products doubles after each cycle of PCR amplification, it is known that the efficiency of amplification decreases with increasing numbers of amplification cycles. The reduced efficiency leads to uneven amplification and loss of proportional quantitative correlation between PCR products and original template numbers (Gause and Adamovicz, 1994). We therefore studied the amounts of PCR products as a function of PCR cycle numbers. The PCR products were measured using ^{32}P-labeled primer, agarose gel electrophoresis and scintillation counting. It was found that the amounts of PCR products were increased logarithmically with the PCR cycle number as expected up to 18 cycles (Figure 2). After 18 cycles, the PCR began to generate less than the expected amounts of amplified products. Therefore, the cycles used for all our quantitative RT-PCR reactions for cytoplasmic HLA-A and -B mRNAs were ≤18.

Next, we studied the PCR products as a function of templates prepared from different quantities of total cytoplasmic RNA. The amounts of RT-PCR products were measured according to fluorescent intensity in agarose gel by scanning densitometry and were linearly correlated with the quantities of templates prepared from 2.5 μg to 15 μg of total cytoplasmic RNA (Figure 3). The results of this experiment indicate that ≤15 μg total cytoplasmic RNA can be used for quantitation of HLA mRNAs.

Separation and Identification of RT-PCR Products of Different HLA-A and -B mRNAs by DGGE

After quantitative amplification, PCR products of the polymorphic region of different specific HLA-A and -B mRNAs were analyzed by DGGE. As shown in Figure 4, this technique successfully separated RT-PCR products of different HLA-A and -B mRNAs. To identify the HLA specificity of each DNA band in denaturing gradient gels, we cloned HLA cDNAs from the same cell lines into plasmids. These plasmids were used

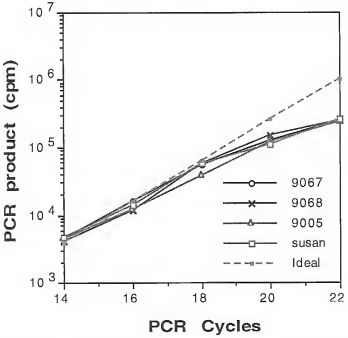


Figure 2 Optimal cycles for quantitative RT-PCR. The amounts of RT-PCR products for HLA-A and -B mRNAs were studied as a function of PCR cycles. The PCR products were measured by using a γ-^{32}P end-labeled primer and scintillation counting of DNA products cut from agarose gels. The ideal relationship between PCR products and PCR cycles is shown as dotted line. Total cytoplasmic RNA (10μg) from four lymphoblastoid cell lines were studied, 9067 (o), 9068 (x), 9005 (Δ) and SH (❑).

as templates for PCR amplification. The PCR products of known HLA specificities were run on the same denaturing gradient gel to determine the identity of each unknown DNA band. As shown in Figure 4B, HLA specificity of each DNA band can be easily identified. When the same approach was applied to three HLA heterozygous LCLs, RT-PCR products of different HLA-A and -B mRNAs were well resolved (Figure 5).

Measurement of Relative Quantities of Different HLA-A and -B mRNAs in The Same Sample by RT-PCR and DGGE

To determine whether RT-PCR and DGGE can be applied to measure the relative quantities of different HLA-A and -B mRNAs in a RNA sample, the following validation study was performed. First, the HLA-A24 and -B60 mRNA transcripts were generated by in vitro transcription from HLA cDNA plasmids and used as templates. The integrity of

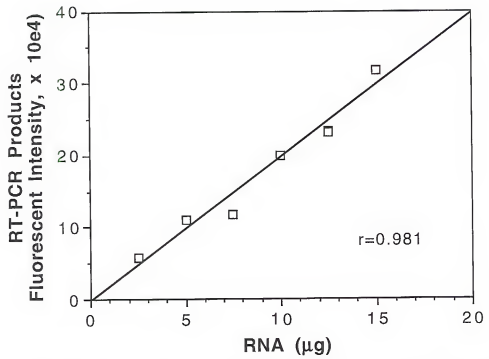


Figure 3 HLA RT-PCR products measured as a function of different amounts of total cytoplasmic RNA. PCR was performed for 18 cycles. The PCR products were identified by agarose gel electrophoresis and ethidium bromide staining, and quantified by densitometry. Simple regression analysis shows a good linear correlation between the two parameters up to 15 µg cytoplasmic RNA.

these transcripts were confirmed by formaldehyde agarose gel electrophoresis which showed that these transcripts were of the expected correct size. The purified transcripts were quantified by absorbance at 260 nm wavelength. These two RNA transcripts were mixed in different ratios (4:1, 2:1, 1:1, 1:2,1:4) at a total amount of 1.5 ng and used for RT-PCR. The results shown in Figure 6 demonstrate that the relative amounts of RT-PCR products for HLA-A24 and -B60 mRNAs were linearly correlated with the relative quantities of the mRNA standards in the reverse transcription mixtures. The same results were obtained when different HLA mRNAs were used (data not shown). These results indicate that RT-PCR/DGGE and phosphor imaging quantitation can be used to determine the relative quantities of different HLA-A and -B mRNAs in the same sample.

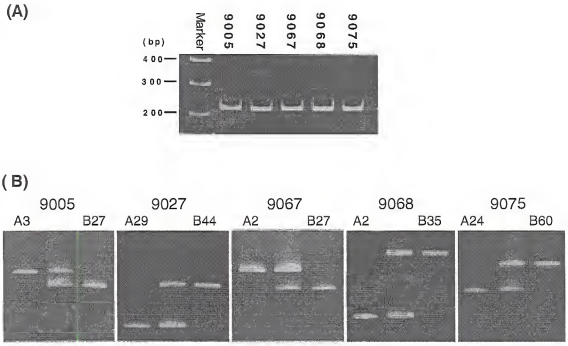


Figure 4 Identification of RT-PCR products of different HLA-A and -B mRNAs by DGGE. (A) Polyacrylamide gel (6%) electrophoresis of RT-PCR products of HLA-A and -B mRNAs from five different HLA-A and -B homozygous LCLs. The identification number of each cell line is shown at the top of each lane. (B) Separation of the RT-PCR products of each cell line in DGGE polyacrylamide gel (middle lane of each small panel). After staining with ethidium bromide, HLA specificity of each DNA band in the PCR products was identified using the PCR product generated from the plasmid containing HLA-cDNA of known specificity from the same cell line (left and right lanes of each small panel).

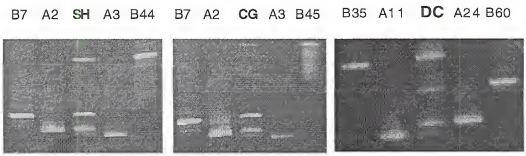


Figure 5 DGGE separation of RT-PCR products of HLA-A and -B mRNAs isolated from LCLs carrying heterozygous HLA-A and -B antigens. The left and the right two lanes of each panel are RT-PCR products from plasmids containing HLA cDNAs of known specificities cloned from the same cell line.

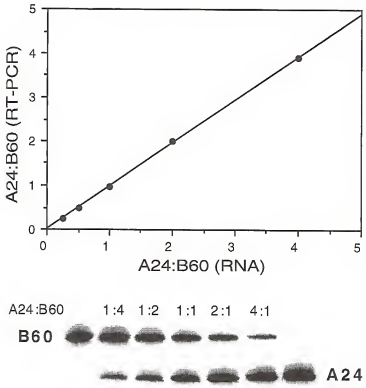


Figure 6 Validation of using RT-PCR and DGGE for measuring relative quantities of different HLA mRNAs. Samples containing different relative amounts of HLA-A24 and -B60 RNA were used as templates for quantitative RT-PCR. These RNAs were synthesized by in vitro transcription. The total amount of HLA-A24 and -B60 RNAs for each RT-PCR reaction was 1.5 ng. The RT-PCR products were separated by DGGE and quantified by phosphor imaging analysis. The bottom panel shows the autoradiograph of the DGGE gel used for quantitative analysis by phosphor imaging.

Measurements of Relative Quantities of Different HLA-A and -B mRNAs in LCLs by RT-PCR/DGGE and S1 Nuclease Protection Assay

To further establish the validity of the RT-PCR/DGGE method, we also determined the relative amounts of HLA-A and -B mRNAs in four LCLs using S1 nuclease protection assay. The results were compared with those obtained by using the RT-PCR/DGGE method. Standards for S1 nuclease protection assay were prepared from the HLA-cDNAs cloned in plasmids. Representative results of using S1 nuclease protection assay for quantitation of HLA-A24 and -B60 mRNAs are shown in Figure 7. We then determined the relative quantities of individual HLA-A and -B mRNAs in four lymphoblastoid cell lines. The results summarized in Table 1 show a good correlation between two assays.

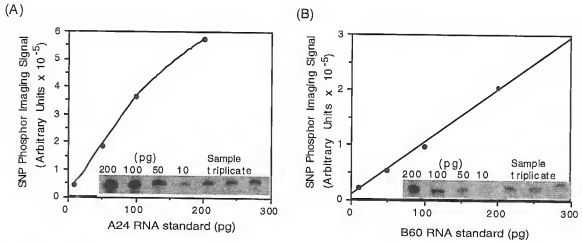


Figure 7 Quantitation of HLA-A24 and -B60 mRNAs in 9075 lymphoblastoid cell line using S1 nuclease protection assay. Panel (A) and (B) show the standard curves for the quantitation of HLA-A24 and -B60 mRNAs. The insets are autoradiographs of standards and triplicates of a RNA sample from 9075 cell line. The same amount of total cytoplasmic RNA was used for quantitation of HLA-A24 and -B60 mRNAs. The amounts of HLA-A and -B mRNAs were determined from the standard curves and used to calculate their relative quantities.

Table 1 Relative quantities of HLA-A and -B mRNAs in lymphoblastoid cell lines measured by RT-PCR/DGGE and S1 nuclease protection assay

LCL	HLA	Relative Quantities of HLA-A and -B mRNAs (%, Mean ± SD) RT-PCR/DGGE Assay	S1 Nuclease Protection
9027	A29	63 ± 4 (4)*	64 ± 11 (2)
	B44	37 ± 4 (4)	36 ± 11 (2)
9067	A2	63 ± 5 (4)	62 ± 4 (2)
	B27	37 ± 5 (4)	38 ± 4 (2)
9068	A2	60 ± 2 (4)	58 ± 1 (2)
	B35	40 ± 2 (4)	42 ± 1 (2)
9075	A24	41 ± 6 (4)	38 ± 6 (3)
	B60	59 ± 6 (4)	62 ± 6 (3)

*: Number of experiments performed on different dates.

Discussion

For quantitation of different specific mRNAs, the commonly used methods include northern blot, S1 nuclease or ribonuclease protection assay, and quantitative RT-PCR (Wiesner and Zak, 1991). The advantages of using northern blot approach are that the integrity of mRNA can be assessed and several rounds of hybridization can be performed using the same blot. However, this method is semi-quantitative and not sensitive. The northern blot method is also complicated by the problem of cross hybridization resulting from high degree of sequence homology and the same size of HLA mRNAs. The second approach to quantify specific mRNAs is S1 nuclease or ribonuclease protection assay. This method is based on solution hybridization and is more sensitive and precise than the northern blot technique. Nevertheless, this approach requires laborious preparation of specific probes and RNA standards. The involvement of several rounds of nucleic acid precipitation by ethanol also introduces variability. The third approach to measure quantities of different HLA mRNAs is quantitative RT-PCR (Gause and Adamovicz, 1994). This is a simple and sensitive quantitative method. The method can be used for large numbers of samples. However, RT-PCR methods alone can not be applied for quantitation of different specific mRNAs that would produce the same size of PCR products in the same incubation. We therefore developed a simple and precise method for measuring the relative quantities of different HLA-A and -B mRNAs in cells using a combined approach involving quantitative RT-PCR, DGGE and phosphor imaging.

The use of quantitative RT-PCR allowed us to have a sensitive technique to amplify different target HLA mRNAs in the same PCR incubation. In order to ensure that cDNA templates of different HLA-A and -B mRNAs were proportionally amplified, the same pair of primers was used for all HLA-A and -B cDNAs. The DGGE technique (Myers et al., 1985) then was used to separate the same size of the amplified PCR products according to minor differences of their nucleotide sequences. The amount of PCR product of each

specific HLA mRNA is quantified by phosphor imaging technique. Although the single strand conformation polymorphism (SSCP) technique (Orita et al., 1989) is another powerful method to separate RT-PCR HLA products, we did not adopt this technique because DNA bands separated in gels of SSCP can not be visualized by simple ethidium bromide staining and incomplete denaturation of DNA samples prior to SCCP gel electrophoresis could introduce quantitative imprecision.

According to the results of our study, the amount of PCR product for each specific HLA mRNA after 18 cycles of amplification appeared sufficient to be detected by ethidium bromide staining (Figs. 3 and 4). Nevertheless, the ethidium bromide staining method and densitometry were not used for quantitation of RT-PCR products separated in DGGE gels. The reason for not using this simple quantitative method is that the same amounts of PCR products for different HLA mRNAs are not equally stained by ethidium bromide in DGGE gels (data not shown). This finding of differential staining likely resulted from different degrees of DNA denaturation in DGGE gels. Consequently, the same amounts of DNA do not bind the same quantities of ethidium bromide. We therefore used primers end-labeled with γ-^{32}P for PCR and phosphor imaging for quantitation of each specific PCR product in DGGE gel.

After optimizing our assay condition, two different approaches were used to validate the RT-PCR/DGGE method for quantitation of different specific HLA-A and -B mRNAs in cells. The first approach was to study samples containing known amounts of different HLA-A and -B RNA transcripts (Figure 6). The second approach was to confirm the results of our method by S1 nuclease protection assay (Figure 7). The results of these two approaches (Figure 6 and Table I) indeed support the validity of our assay method. However, our method, unlike an S1 nuclease protection assay, yielded relative but not absolute quantities of different HLA-A and -B mRNAs. If absolute amounts of specific HLA mRNAs need to be determined, a separate measurement of total HLA-A and -B mRNAs can be made by additional quantitative RT-PCR (Gilliland et al., 1990;

Prendergast et al., 1992). The exact amount of each specific HLA-A or -B mRNA then can be calculated from the results of relative quantities of different specific HLA mRNAs and the total HLA mRNAs. Therefore, the newly established RT-PCR/DGGE method will be useful to study the mechanisms of normal or altered differential quantitative expression of HLA-A and -B antigens in normal and neoplastic cells under different physiological or pathological conditions (i.e. cytokine stimulation, viral infection and malignant transformation). Because there are many other duplicated and highly conserved genes (Greig et al., 1993; Li et al., 1995; Spicer et al., 1995; Watkins, 1995), the results our study also demonstrate that the RT-PCR/DGGE method should be useful for studying the differential quantitative expression of these genes in cells.

In view of the extreme polymorphic nature of class I HLA antigens, we expect that the protocol described in this report may not resolve certain combinations of HLA phenotypes due to limited differences in nucleotide sequences (e.g. HLA-B60 and B61). If this situation occurs, a different set of primers can be selected for RT-PCR, and DGGE conditions (i.e. gradients of denaturing agent and/or temperature of gel electrophoresis) can be modified to resolve the PCR products of different HLA mRNAs. In addition to the eight cell lines studied by us (Figure 4 and 5), we have successfully used the protocol described in this report to determine the relative quantities of different HLA-A and -B mRNAs in three additional HLA-phenotyped LCLs. These three cell lines are 9001 (A24 and B7), 9003 (A24 and B51), and 9044 (A24, B51 and B63). However, we were unable to resolve HLA-A2/A11 and B60/B61 in two additional cell lines by our protocol without modification. Because HLA-A and -B antigens that have been studied by us are present in relatively high frequencies in the general population, the results of our study indicate that the protocol reported herein should be applicable to many HLA-phenotyped cells. By using this validated approach, we are able to measure the relative quantities of HLA-A and -B mRNAs in subsequent studies as described in the next chapter.

CHAPTER 3
MECHANISMS FOR DIFFERENTIAL QUANTITATIVE EXPRESSION OF HLA-A AND -B ANTIGENS IN LYMPHOBLASTOID CELL LINES

Introduction

As discussed in Chapter 1, class I HLA molecules are polymorphic membrane glycoproteins and consist of a 44-kD heavy chain encoded by the classical class I HLA genes (HLA-A, -B, and -C) and a 12-kD invariant light chain (β2m) encoded by a non-MHC gene (Bjorkman and Parham, 1990). The allelic polymorphism of heavy chains is responsible for different peptide-binding specificities of class I HLA molecules and is functionally important in providing HLA-restricted immune responses (Bjorkman and Parham, 1990).

Previous studies have shown that different class I HLA genes are differentially expressed, and the relative quantities of HLA-A and -B antigens expressed in different types of cells are the same in an individual (Kao and Riley, 1993). The relative quantities of HLA-A and -B antigens expressed on cells remain constant over time (Kao, 1989; Shieh and Kao, 1995). Subsequent studies of comparing the quantitative expression of different specific HLA antigens in members of HLA phenotyped families indicated that the differential quantitative expression of HLA-A and -B antigens is linked directly to class I HLA genes and follows Mendelian inheritance (Kao and Riley, 1993). In addition, it was found that the relative amounts of different specific HLA antigens are proportionally amplified during up-regulated expression of total class I HLA antigens induced by interferon treatment or infection with influenza virus (Shieh and Kao, 1995). Because the amount of HLA antigens expressed on cells has been shown to determine the susceptibility

of cells to cytotoxic T lymphocytes (Shieh et al., 1996), these findings support the potential functional importance of genetically predetermined quantitative HLA expression in determining the susceptibility of cells to cytotoxic T cells, which may influence the morbidity or recovery of an individual from various infection by intracellular pathogens. For this reason, it would be important to gain further understanding of how differential expression of HLA antigens is regulated.

The quantitative HLA protein expression in cells is determined by the rates of protein synthesis and degradation, and the synthesis of protein is regulated by the amount of available HLA-mRNA and the protein translation efficiency. For HLA expression, it is also determined by availability of antigen peptides and β2m for correct folding and stabilization. However, available results support that the availability of antigenic peptides and β2m is not the rate-limiting step in normal cells (Shieh and Kao, 1995). Although numerous studies on regulation of HLA expression have been reported (Bishara et al., 1988; Blanar et al., 1989; Driggers et al., 1990; Gerrard et al., 1988; Girdlestone and Milstein, 1988; Hakem et al., 1989; Masucci et al., 1987; Versteeg et al., 1989a; Zachow and Orr, 1989), most of them have only focused on the transcription step affected by variations in the promoter sequences of different HLA-A and -B genes. Studies also showed that certain sequence variations in the introns contribute to the varied quantitative expression of certain class I HLA antigen (Laforet, 1997; Magor et al., 1997). Although the results of these studies indicate that sequence variations of HLA genes could directly influence protein expression, most of these studies have been conducted by using tumor cell lines or cells transfected with class I HLA cDNA constructs or genes. Therefore, results obtained from these studies are insufficient and do not necessarily explain the molecular basis of genetically pre-determined quantitative differential expression of HLA antigens in human cells. For this reason, we decided to study how different regulatory steps of protein expression are involved in determining the quantitative differential expression of HLA antigens.

Because earlier studies have shown that the relative quantities of different HLA-A and -B antigens in EBV-transformed lymphoblastoid cell lines are the same as their parental B lymphocytes (Shieh and Kao, 1995), and homozygous cell lines with well characterized HLA phenotypes are readily available (Prasad and Yang, 1996; Yang et al., 1989), we chose EBV-transformed lymphoblastoid cell lines for our study. The study described herein shows that different steps, including transcription, splicing, mRNA degradation, and possibly translation, are involved in regulation of the expression of each HLA antigen. Despite the complex regulatory mechanisms for HLA expression, the results of our study showed that HLA gene sequences are responsible for all the studied regulatory steps and the differential quantitative expression of HLA antigens is directly determined by HLA genes.

Materials and Methods

Lymphoblastoid Cell Lines and RNA Preparation

EBV-transformed LCLs with well characterized class I HLA phenotypes were obtained from American Society for Histocompatibility and Immunogenetics Cell Repository (Prasad and Yang, 1996; Yang et al., 1989) or developed in our laboratory (Shieh and Kao, 1995). These cell lines were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% fetal calf serum, 1% antibiotic-antimycotic solution and 40 μg/ml gentamycin.

Isolation of Nuclei and Preparation of RNA

The nuclei of lymphoblastoid cells were prepared by using the method described by Mullner et al. (1997). Briefly, 200 million cells were lysed in 12 ml of lysis buffer containing 150 mM sucrose, 0.25 mM EGTA, 1 mM EDTA, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES pH 7.5, 14 mM β-

mercaptoethanol and 0.2% NP-40. The homogenate was diluted with 12 ml of buffer II, which contains 2 M sucrose, 0.25 mM EGTA, 1 mM EDTA, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES pH 7.5 and 14 mM β-mercaptoethanol. This diluted homogenate was layered over a cushion of buffer II representing 1/3 of the volume of the centrifuge tube. After centrifugation at 30,000g for 45 min at 4°C in a rotor with swing-out buckets, the supernatant containing the cytoplasm was saved for isolation of cytoplasmic RNA. The sucrose layer was removed and the pellet containing nuclei was resuspended in the storage buffer containing 20 mM Tris-HCl pH 8.0, 75 mM NaCl, 0.5 mM EDTA, 50% glycerol, 0.85 mM DTT and 125 mM phenylmethylsulfonyl fluoride (PMSF) at a concentration of 1 x 10^5 nuclei/μl. Total nuclear RNA and cytoplasmic RNA were isolated using the RNAeasy Total RNA Kit (QIAGEN Inc., Chatsworth, CA) according to the manufacturer's protocol. Remnant DNA in the RNA preparation was removed by RNase-free DNase I digestion for 1 hr at 37°C.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) of Nuclear RNA

The reverse transcription was performed as described in Chapter 2 except that 0.5 μg total nuclear RNA was used for each reverse transcription reaction. Five microliters of the reverse transcription products were used as templates for PCR as described in Chapter 2 for 26 cycles using primers designed based on the sequences of HLA-A and -B exon 2 and intron 2. In this condition, PCR amplification efficiency is still in the linear range. The primer sequences are 5'-GCT CCC ACT CCA TGA GGT ATT TC-3' and 5'-GAA AAT GAA ACC GGG TAA AGG CGC-3'. The PCR products were separated by agarose gel electrophoresis and isolated by using Quick Gel Extraction Kit (QIAGEN Inc., Chatsworth, CA). Two nanograms of these PCR products were then used as templates for a second round PCR of 8 cycles, which amplifies the exon 2 sequences in the linear range of amplification efficiency. The primer sequences for the second round PCR are 5'- GCT CCC ACT CCA TGA GGT ATT TC -3' and 5'- CCT CGC TCT GGT TGT AGT AGC -

3'. Therefore, these PCR products are generated only from the HLA-A and -B transcripts containing intron 2. Another PCR was also performed to amplify the HLA-A and -B transcripts in which intron 2 has been spliced. The primer sequences for this PCR are 5'-CGC CGT GGA TAG AGC AGG-3' (nucleotides 218-235 in exon 2) and 5'- GCG ATG TAA TCC TTG CCG-3' (nucleotides 429-446 in exon 3).

Determination of The Relative Quantities of cytoplasmic HLA-A and -B mRNAs Using RT-PCR/DGGE and Phosphor Imaging

The method is the same as that described in details in Chapter 2. Briefly, first-strand HLA cDNAs were prepared by reverse transcription of 10 μg of total cytoplasmic RNA using the primer (5'-ACA GCT CC(A,G) (A,G)TG A (C,T)C ACA-3') which is specific and complementary only to the nucleotide sequences of exon 5 of all class I HLA-A and -B genes, but not to those of HLA-C genes. After inactivation of reverse transcriptase by heating at 99°C for 5 min, the HLA-A and -B cDNAs were used for quantitative RT-PCR to determine the relative amounts of different HLA-A and -B mRNAs by DGGE and phosphor imaging analysis.

The nucleotide sequences of the primers used for quantitative PCR in amplifying the coding sequence 218-446 are 5'-CGC CGT GGA TAG AGC AGG-3' and 5'-GCG ATG TAA TCC TTG CCG-3', which are complementary to the conserved antisense and sense nucleotide sequences in exon 2 and exon 3 of all class I HLA mRNAs, respectively. The amplified products encompass the coding sequence 218-446. Quantitative PCR was performed for 18 cycles. One primer was end-labeled with [γ-^{32}P]ATP, and the HLA-A and -B cDNA prepared from cytoplasmic RNA was used as templates.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed in 1 mm thick 6% polyacrylamide (acrylamide: bisacrylamide = 19:1) gel using D-GENE™ Denaturing Gradient Gel Electrophoresis

System (Bio-Rad Laboratories, Hercules, CA). The polyacrylamide gel contained a linearly increasing denaturant gradient from 40% to 60%. The 100% denaturant contains 7 M urea and 40% (w/v) deionized formamide. Electrophoresis was performed at 60°C, 165 V for 1.5 to 3.5 hours in 40 mM Tris-acetate, pH 8.0, containing 1 mM EDTA (TAE). After electrophoresis, the gels were dried for autoradiography and phosphor imaging analysis. PCR product of each HLA-A or -B mRNA in DGGE gel was identified by using PCR products prepared from the plasmids containing cloned HLA-cDNAs of known specificity.

IEF-PAGE and Immunoblotting for Measuring The Relative Quantities of HLA-A and -B Antigens in LCLs.

One million EBV-transformed lymphoblastoid cells were solubilized in 2 ml Triton X-114 (TX-114) (Sigma, St. Louis, MO, USA) containing buffer at 4°C. After phase separation of TX-114 detergent at 37°C, the extracted membrane proteins in TX-114 detergent phase were treated with 300 µl of neuraminidase (2.5 U/ml, Type X; Sigma) at 37°C for 6 hours under constant mixing in a Thermomixer 5436 (Eppendorf, Hamberg, Germany) to avoid phase separation. After neuraminidase digestion the detergent phase was collected and diluted with an equal volume of IEF buffer for IEF-PAGE. After IEF-PAGE, the IEF gels were washed and proteins in the gels were electrophoretically transferred to Immobilon membrane (Millipore, Bedford, MA, USA) at 40 volts for 45 minutes. Thereafter, the Western blots were blocked with 5% nonfat milk and incubated sequentially with 171.4 anti-HLA heavy-chain mAb and alkaline phosphatase conjugate of rabbit anti-mouse IgG antibody. The detailed procedures were reported previously (Kao and Riley, 1993).

Immunoprecipitation of Pulse-chase Radiolabeled HLA Antigens from LCLs

EBV-transformed human lymphoblastoid cell lines (DC, 9001, 9027, 9028, 9067, 9068, 9075) were cultured in RPMI-1640 medium (GIBCO BRL Life Technologies,

Grand Island, NY) containing 10% newborn calf serum (GIBCO BRL), 1% antibiotic-antimycotic solution (Sigma Co., St. Louis, MO), and 0.1% gentamycin (Sigma). Fifteen million lymphoblastoid cells at log phase were harvested and washed twice with 10 ml PBS. The cells were resuspended in 3 ml methionine-free RPMI-1640 with 10% dialyzed FCS. The cell suspension was then incubated with 300 μCi ^{35}S- methionine (Amersham Life Science, Inc., Arlington Heights, IL) at 37°C for 2 hours. Cells were harvested, washed twice with ice-cold PBS, and suspended in 3 ml regular RPMI-1640 with 10% FCS. Three million of these cells were chased with 1 mM unlabeled methionine at 37°C for 0 or 18 hours. The cells were collected and washed twice with ice-cold PBS. These cells were then solubilized with 3 ml TX-114 containing solubilization buffer on ice for 15 min. After centrifugation at 10,000g for 10 min at 4°C , the supernatant was collected and freezed at -70°C until use.

For immunoprecipitation, the harvested supernatant was preincubated with 20 μl of 10% washed staphylococus A (Sigma) suspended in TX-114 containing buffer on ice for 60 min. After removal of staphylococcus A by centrifugation at 10,000g for 5 min at 4°C, the supernatant was incubated with 15 μg of W6/32 anti-HLA monoclonal antibody at 4°C overnight. Thereafter, the immune complexes were precipitated by mixing with 30 μl of 10% staphylococcus A and incubation at 4°C for 2 hr. After centrifugation at 10,000g for 5 min at 4°C, the staphylococcus A pellet was washed sequentially with PBS containing 0.25 M NaCl and 0.1% NP-40, and PBS containing 0.1% NP-40. The washed staphylococcus A pellet was resuspended in 100 μl of 12.5 units/ml type X neuraminidase at pH 6.0 and 37°C for 6 hr. After centrifugation at 10,000g for 10 minutes, the HLA antigens were eluted by resuspending the staphylococcus A pellet in 40 μl of 1x IEF sample buffer with 2-mercaptoethanol and incubation at room temperature for 10 min. After centrifugation at 10,000g for 5 min, the supernatant was harvested and used for IEF-PAGE which is followed by phosphor imaging and autoradiography.

Treatment of LCLs with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB)

Five million of EBV-transformed lymphoblastoid cells were treated with 25 μg/ml DRB in RPMI 1640 complemented with 5% FCS for 0 or 23 hours. Total cytoplasmic RNA was extracted and 2 μg of the RNA was used for reverse transcription as described above in a total volume of 30 μl. The relative quantities of HLA-A and -B mRNAs were determined by quantitative PCR/DGGE and phosphor imaging as described above.

Nuclear run-on

Ten million nuclei of LCLs were incubated for 30 min at 26°C in 250 μl of a buffer containing 83 mM $(NH4)_2SO_4$, 87 mM Tris-HCl (pH 7.9), 4 mM $MgCl_2$, 4 mM $MnCl_2$, 24 mM NaCl, 0.2 mM EDTA, 3 mM PMSF, 0.9 mM DTT, 0.75 mM each NTP, 10 mM creatine phosphate, 0.15 mg/ml creatine phosphokinase and 20% glycerol. The reaction was stopped by adding 50 units of DNase I and incubating for another 2 min at 26°C. Then the denaturing buffer containing guanidinium thiocyanate to be used in RNA preparation was immediately added into the mixture, and the nuclear RNA was extracted as described above.

Autoradiography, phosphor imaging and densitometry

For autoradiography, the gels were exposed to Fuji Medical X-ray film (Fuji Photo Film Co., Ltd., Japan) for 3 hr (DGGE) or to Kodak Biomax MR film (Kodak Scientific Imaging Systems, Rochester, NY) for 48 hr (IEF-PAGE) at -70°C. For phosphor imaging, the DGGE gels were exposed to a Phosphorscreen (Molecular Dynamics, Inc., Sunnyvale, CA) for 3 hours at room temperature. The radioactivity of each specific DNA band was quantified using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). For quantitation of HLA protein by densitometry, immunoblots or X-ray films were scanned with a 600 dpi Microtek grayscale scanner (Microtek, Inc., Torrance, CA) and analyzed by Collage 3.0 software (Fotodyne Inc., New Berlin, WI).

Results

Degradation of Different HLA-A and -B Antigens in Cells

Because the steady state protein levels are determined by rates of degradation and synthesis, we first studied whether different HLA-A and -B antigens are proportionally degraded, after synthesis using pulse-chase experiments in five different lymphoblastoid cell lines. The HLA proteins in cells were pulse-labeled with ^{35}S-methionine for 2 hours and chased with excess amount of cold methionine for 18 hours. The HLA proteins were immunoprecipitated with W6/32 monoclonal antibody, which recognizes the native form of class I HLA antigens. After neuraminidase digestion, different HLA-A and -B proteins were separated by IEF gel electrophoresis. The relative quantities of each ^{35}S-labeled HLA-A and -B protein was measured by autoradiography and densitometry. The results of this study in these five cell lines with homozygous HLA-A and -B antigens show that the relative quantities of ^{35}S-methionine labeled HLA-A and -B proteins remain similar after 18 hr chase with cold methionine (Figure 8). The differences of relative amounts of HLA-A and -B antigens before and after cold chase were within the experimental variation, and were not significant. Thus, this finding indicated that different specific HLA-A and -B proteins have similar stabilities and are degraded proportionally.

The Relative Quantities of Different HLA-A and -B Proteins Generally Are Proportionally Correlated with That of Their mRNAs.

Because different HLA-A and -B proteins have similar degradation rates in these studied cell lines, we then determined whether the relative quantities of different HLA-A and -B proteins are correlated with those of their respective mRNAs. The relative quantities of different HLA-A and -B proteins were measured by IEF gel electrophoresis, immunoblotting and densitometric analysis (Kao and Riley 1993). The relative quantities of their mRNAs were determined by RT-PCR/DGGE and phosphor imaging (Liu and Kao 1997). The results shown in Table 2 indicate that the relative quantities of different HLA-A

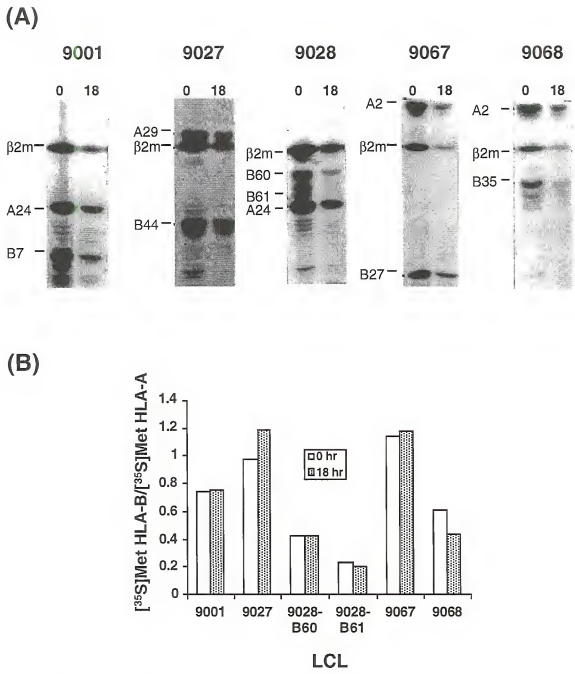


Figure 8 Turnover of ^{35}S-methionine-labeled HLA-A and -B proteins in lymphoblastoid cell lines (LCLs). (A) Autoradiographs of 1D-IEF gel. EBV-transformed lymphoblastoid cell were pulse-labeled with [^{35}S]methionine for 2 hours and chased with cold methionine for 0 hour (**0**) or 18 hours (**18**). The immunoprecipitated HLA proteins were separated by IEF gel electrophoresis. (B) The relative quantities of [^{35}S]methionine-labeled HLA-A and -B proteins before and after 18-hour cold methionine chase. The quantities of [^{35}S]methionine-labeled HLA-A and -B proteins were determined by scanning densitometry.Each bar represents the mean value of two experiments. The variation between these two experiments is less than 15%.

Table 2 Relative quantities of HLA proteins and mRNAs in ten different lymphoblastoid cell lines (LCLs)

LCL	Phenotype	Relative Quantity of Protein¶ (%) (Mean±SD) (n)	Relative Quantity of mRNA¶ (%) (Mean±SD) (n)
9005	A3	36 ± 6 (5)*	35 ± 5 (4)*
	B27	64 ± 6 (5)	65 ± 5 (4)
9027	A29	61 ± 5 (9)	63 ± 4 (4)
	B44	39 ± 5 (9)	37 ± 4 (4)
9067	A2	62 ± 6 (6)	63 ± 5 (4)
	B27	38 ± 6 (6)	37 ± 5 (4)
9068	A2	64 ± 3 (4)	60 ± 2 (4)
	B35	36 ± 3 (4)	40 ± 2 (4)
SH	A2	28 ± 7 (4)	48 ± 3 (2)
	A3	20 ± 6 (4)	13 ± 1 (2)
	B7+B44§	52 ± 10 (4)	B7 27 ± 1 (2)
			B44 13 ± 2 (2)
CG	A2-var	22 ± 4 (4)	41 ± 1 (2)
	A3	15 ± 7 (4)	15 ± 1 (2)
	B7	47 ± 7 (4)	28 ± 1 (2)
	B45	16 ± 4 (4)	15 ± 1 (2)
9075	A24	63 ± 3 (5)	41 ± 6 (4)
	B60	37 ± 3 (5)	59 ± 6 (4)
DC	A11	25 ± 4 (5)	13 ± 3 (5)
	A24	48 ± 4 (5)	26 ± 3 (5)
	B35	14 ± 4 (5)	28 ± 3 (5)
	B60	14 ± 2 (5)	31 ± 7 (5)
9001	A24	51 ± 5 (3)	42 ± 6 (4)
	B7	49 ± 5 (3)	58 ±6 (4)
9028	A24	67 ± 8 (3)	40 ± 3 (2)
	B60+B61†	33 ± 8 (3)	60 ± 3 (2)

¶: The relative quantities of HLA-A and -B proteins were measured by IEF gel electrophoresis, immunoblotting and scanning densitometry. The relative quantities of HLA-A and -B mRNAs were measured by quantitative RT-PCR/DGGE and phosphor imaging. Relative quantity of HLA-A or -B = (quantity of HLA-A or -B/(quantity of HLA-A + quantity of HLA-B)) x 100%.
*: Number of independent measurements.
§: HLA-B7 and-B44 proteins can not be separated by IEF-PAGE. They were quantified together.
†: RT-PCR products of HLA-B60 and B61 mRNAs can not be separated by DGGE. They were measured together.

and -B proteins are proportional to those of their mRNAs in the 9005, 9027, 9067 and 9068 cell lines. since different HLA proteins have similar stability, these results suggest that in these cell lines different HLA-A and -B mRNAs have similar protein synthesis rates. However, the relative quantities of HLA-A and -B proteins are not proportionally correlated with their mRNAs in HLA-A24 positive cell lines (9028, 9075 and DC). It appears that proteins are expressed in higher quantities relative to their mRNA transcripts in these cell lines. This phenomenon was also observed in HLA-B7 positive cell lines (SH and CG) but not in the 9001 cell line, which is homozygous for HLA-A24 and -B7. These results suggest that both HLA-A24 and -B7 mRNAs may be more efficient in synthesizing HLA proteins. This possibility is further substantiated by the predominance of HLA-A24 and -B7 protein bands on IEF gel (Figure 9). A relative large number of HLA-A24 positive cell lines were studied in order to substantiate our initial observation.

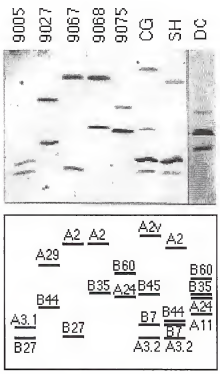


Figure 9 IEF-immunoblot of HLA-A and -B antigens from nine different lymphoblastoid cell lines. The lower diagram shows the specificities of these antigens. The relative amounts of HLA proteins are determined by scanning densitometry.

Different HLA-A and -B mRNAs Have Similar Stabilities

Because the steady state of HLA mRNA is regulated by both HLA mRNA production and degradation, whether different HLA-A and -B mRNAs have the same stabilities could be a factor in determining their differential quantities. By using DRB to inhibit mRNA synthesis, we performed HLA mRNA degradation studies in seven lymphoblastoid cell lines. First we studied the inhibition of HLA mRNA as a function of different concentrations of DRB (Figure 10A) and found that 25 μg/ml of DRB can maximally inhibit HLA mRNA synthesis. We also conducted a time course study (Figure 10B) and found that, in order to detect significant degradation of HLA mRNA, 24-hour incubation is sufficient. On the basis of these studies, we studied the relative quantities of HLA-A and -B mRNAs after 23-hour treatment with 25 μg/ml of DRB. The relative quantities of HLA-A and -B mRNAs in these cells before and after DRB treatment were measured by using RT-PCR/DGGE and phosphor imaging. The results summarized in Figure 11 show that different HLA-A and -B mRNAs are proportionally degraded in five of the seven studied cell lines. A slight difference between the stabilities of HLA-A and -B mRNAs was noted in the 9027 and 9067 cell lines. Our results indicated mRNA stability is not a major factor influencing the differential expression of HLA-A and -B antigens in majority of cell lines. However, this mechanism is optional in some cell lines.

Pre-mRNA Splicing Is An Important Factor Determining The Quantitative Differential Expression of HLA-A and -B Antigens

Because different HLA-A and -B mRNAs have similar stabilities in many LCLs, it is likely that HLA-A and -B mRNAs are differentially produced. The mRNA production rates are determined by transcription and/or pre-mRNA splicing rates. Our original plan was to use PCR-based nuclear run-on to determine whether HLA-A and -B genes are differentially transcribed. Isolated nuclei were incubated with or without NTPs. Then nuclear RNA was extracted and RT-PCR was performed to amplify the unspliced transcripts. The difference of the measurements from these two incubations should

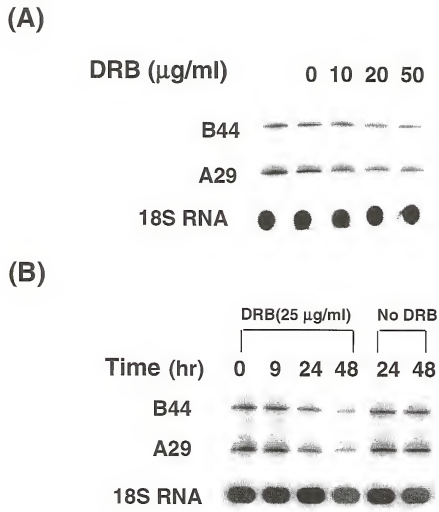


Figure 10 The effect of DRB treatment on HLA mRNA levels. (A) Treatment of 9027 LCL with different concentrations of DRB for 23 hours. After treatment of cells with different concentrations of DRB, two micrograms of total cytoplasmic RNA were used as template for Quantitative RT-PCR to amplify HLA-A and -B mRNAs. The RT-PCR products of HLA-A and -B mRNAs were separated by DGGE and quantified by phosphor imaging. The dot blot of 18S ribosomal RNA in 0.2 μg of each RNA sample was also performed to determine whether RNA from same number of cells were used in RT-PCR. (B) Treatment of 9027 LCL with 25 μg/μl of DRB for different times. After treatment of cells with different concentrations of DRB, two micrograms of total cytoplasmic RNA were used as template for Quantitative RT-PCR to amplify HLA-A and -B mRNAs. The RT-PCR products of HLA-A and -B mRNAs were separated by DGGE and quantified by phosphor imaging. The slot blot of 18S ribosomal RNA in 0.2 μg of each RNA sample was also performed to determine whether RNA from same number of cells were used in RT-PCR.

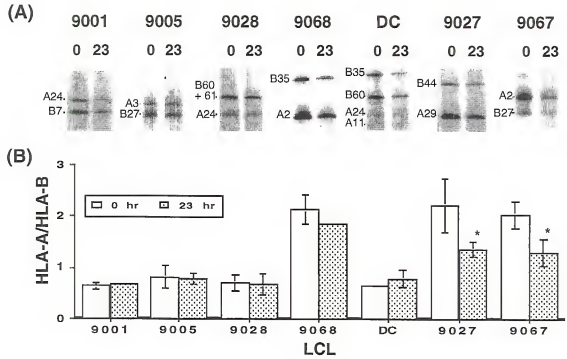


Figure 11 Turnover of HLA-A and -B mRNAs in LCLs. (A) Phosphor images of DGGE gels for measuring the relative quantities of HLA-A and -B mRNAs before and after 23 hour DRB treatment. (B) Ratios (mean±SD) of HLA-A to -B mRNAs in different lymphoblastoid cell lines before and after 23 hr inhibition with 25 µg/ml of DRB. Each value represents the mean of two or three separate measurements were performed for each determination. *: $p < 0.05$.

represent the newly synthesized transcripts. The results of PCR-based nuclear run-on from 4 cell lines shows that there was only a small increase in the amount of unspliced transcripts (Figure 12). The difference represents less than 20%, suggesting a relatively large pre-existing pool of unspliced HLA transcripts. This observation was further supported by the results from RT-PCR of nuclear RNA, in which the sequences spanning exon 2 and exon 3 were amplified (Figure 13). Due to this large pre-existing pool, we were unable to reliably study the differential transcription of HLA genes by using the PCR-based nuclear run-on approach. The results also suggest that HLA pre-mRNA splicing could be a rate-limiting step in regulating the differential HLA mRNA production. To investigate whether the relative quantities of different HLA-A and -B transcripts are determined by splicing, transcription, or both, we decided to measure the relative amounts

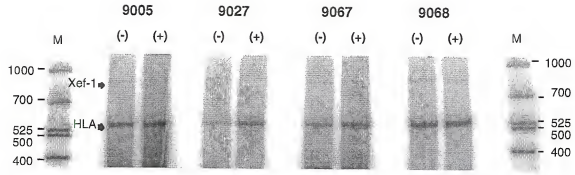


Figure 12 PCR-based nuclear run-on in four LCLs. Nuclear run-on reaction with (+) or without (-) NTPs, the HLA-A and -B nuclear transcripts were used for quantitative RT-PCR. The PCR primers, 5'- TGG GCG GGT GAG TGC GGG GTC-3' and 5'-GAA AAT GAA ACC GGG TAA AGG CGC-3', correspond to the sequences of HLA gene in intron 1 and intron 2, respectively. In vitro synthesized Xef-1 mRNA added before RNA extraction as an exogenous control was also quantified by RT-PCR. M: DNA markers.

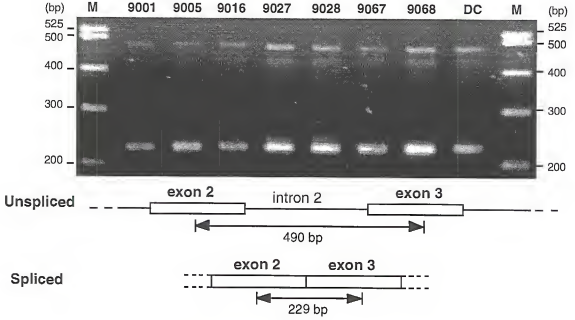


Figure 13 Presence of abundant unspliced HLA transcripts in nuclei. Agarose gel electrophoresis shows two sizes (229 bp and 490 bp) of RT-PCR products from HLA nuclear transcripts. The light bands (sightly shorter than 490 bp) are likely the RT-PCR products of unspliced nuclear HLA transcripts with shorter intron 2. Sequences of the PCR primers correspond to sequences of exon 2 and 3 of HLA genes.

of HLA-A and -B transcripts before and after splicing in nuclei. The relative quantities of HLA-A and -B transcripts in cytoplasm and nuclei were determined by RT-PCR/DGGE

and phosphor imaging. Three different groups of transcripts were studied (Figure 14). The first group are the prespliced transcripts with intact intron 2 in nuclei (Group I). The

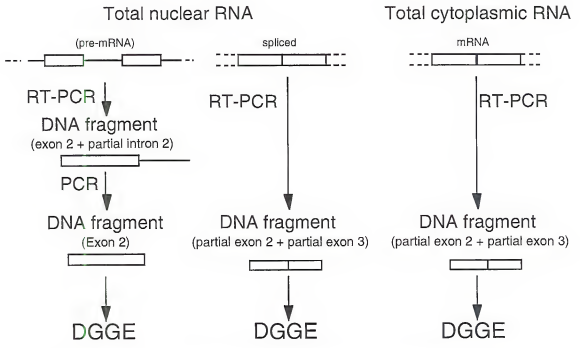


Figure 14 Experimental design for study of HLA mRNA production.

second group of transcripts are the spliced transcripts without intron 2 in nuclei (Group II). The third group are the mature mRNA transcripts in cytoplasm (Group III). Because we were able to separate RT-PCR products generated from exon 2 or exon 2 and exon 3 of HLA-A and -B transcripts by using DGGE, this approach provided us with a simple way to study whether pre-mRNA splicing plays any critical role in determining the quantitative differential expression of HLA-A and -B antigens. To amplify the first groupof HLA-A and -B transcripts in which intron 2 has not been spliced, we first used a pair of primers complementary to the 5' end of exon 2 and a sequence in intron 2. The amplified products were generated from the transcripts containing intron 2. These PCR products were purified and used as template for second round of PCR to amplify the HLA-A and -B exon 2 sequences, which were separated by DGGE and quantified by phosphor imaging. For

amplifying Group II and Group III of HLA-A and -B transcripts, the protocols described in chapter 2 are used, in which the PCR products only include partial exon 2 and partial exon 3. The results of this study in nine lymphoblastoid cell lines are shown in Figure 15.

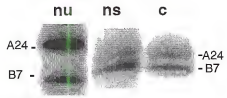
Our results show that in cell lines 9027, 9067, the relative quantities of HLA-A and -B transcripts are the same for all three groups of transcripts. For the 9005 cell line, which is homozygous for HLA-A3 and -B27, the relative quantities of HLA-A3 transcripts prior to splicing is greater than that of spliced transcripts in nuclei and cytoplasm. This finding suggest unequal rates of splicing for HLA-A and -B mRNAs (Figure 15). The same finding was obtained for all A24-positive cell lines (9001, 9028, 9075, and DC). Our results demonstrated that nuclear splicing of HLA pre-mRNAs could play a major role influencing the quantitative differential expression of HLA-A and -B antigens.

Discussion

The primary purpose of this study is to determine the roles of gene transcription, splicing, mRNA turnover and translation in regulation of genetically pre-determined differential expression of different HLA-A and -B antigens. The results of our studies show that regulation of the quantitative differential expression of different HLA-A and -B antigens is determined by combinations of multiple steps that include HLA gene transcription, pre-mRNA processing, mRNA turnover and/or mRNA translation. In all of these steps, gene transcription and pre-mRNA processing appear to play the major roles for majority of different HLA-A and -B antigens. Turnover and translation of HLA mRNAs are involved for a few specific HLA-A and -B alleles. Despite the complexity of regulatory mechanisms for HLA expression, all are directly linked to the coding and noncoding nucleotide sequences of HLA genes. This finding supports an earlier report that differential quantitative expression is directly linked to HLA genes and follows Mendelian laws (Kao and Riley, 1993).

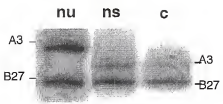
Figure 15 Measurements of the relative quantities of nuclear and cytoplasmic HLA-A and -B transcripts by using quantitative RT-PCR/DGGE and phosphor imaging in seven lymphoblastoid cell lines. **nu**: unspliced nuclear HLA-A and –B transcripts. **ns**: spliced nuclear HLA-A and -B transcripts. **c**: cytoplasmic HLA-A and –B mRNAs. Each value represents the mean±SD of three separate experiments. *: Two separate experiments for 9075 cell line.

9001



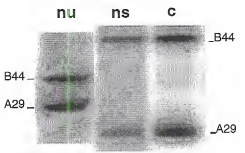
A24(%)73±7 36±4 45±6
B7(%) 27±7 64±4 55±6

9005



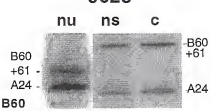
A3(%) 51±6 36±4 34±3
B27(%) 49±6 64±4 66±3

9027



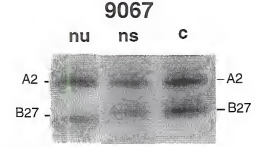
B44(%) 43±1 49±1 47±3
A29(%) 57±1 51±1 53±3

9028



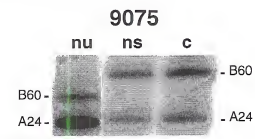
B60
+61(%) 25±16 45±4 55±6
A24(%) 75±16 54±4 45±6

9067



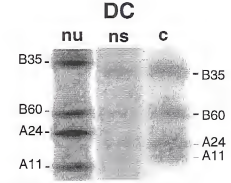
A2(%) 59±15 55±1 54±5
B27(%) 41±15 45±1 46±5

9075



B60(%) 25±1* 60±1* 58±2*
A24(%) 75±1* 40±1* 42±2*

DC



B35(%) 22±9 36±5 30±9
B60(%) 19±3 23±3 27±3
A24(%) 33±4 25±2 28±4
A11(%) 25±8 16±1 15±2

In our study, we used the validated RT-PCR/DGGE and phosphor imaging to measure the relative quantities of HLA-A and -B mRNAs (Chapter 2). This approach allows us to avoid complications of cross-hybridization and the variations in specific activity of probes that are frequently encountered in northern blot. For measuring the relative quantities of different specific HLA-A and -B antigens, cytoplasmic mRNAs were used as templates and the primer complementary to a sequence in exon 5 shared by HLA-A and -B mRNAs were used to prepare cDNAs. This sequence encodes part of transmembrane domain of all HLA-A and -B proteins. Therefore, only the relative quantities of the HLA mRNAs encoding the whole length HLA transmembrane heavy chains were measured. For measuring the relative amounts of different HLA-A and -B proteins, the use of TX114 to solubilize cells, which only extracts the transmembrane proteins (Bordier, 1981), allows us to quantify the relative amounts of intact transmembrane HLA-A and -B antigens by IEF-PAGE and immunoblotting. The aforementioned two approaches made it possible for us to determine whether the relative amounts of different HLA-A and -B antigens are proportionally correlated with those of their mRNAs. The results shown in Table 2 indicate that, for most of the studied LCLs, HLA-A and -B protein levels are proportionally correlated with their mRNA levels, except for those positive with HLA-A24 or -B7. Because different HLA proteins have similar turnover rates (Figure 8), these results indicated that rates of mRNA production play important roles in determining HLA protein levels. In addition, the results suggest that HLA-A24 and -B7 mRNAs are more efficient in protein translation and that differential translation of mRNAs for certain HLA antigens plays a role in determining HLA expression.

Next, we studied the role of stability of different HLA-A and -B mRNAs in influencing the quantitative differential expression of different HLA-A and -B antigens. This study was accomplished by measuring changes of relative quantities of different HLA-A and -B mRNAs before and after treatment of cells with DRB, an inhibitor of RNA

polymerase II, for 23 hours. The results showed that HLA-A and -B mRNAs are proportionally degraded in five out of seven cell lines studied. Similar observation was made previously by other inhibitors using HLA-A and -B transgenes (McCutcheon et al., 1995). However, our study showed that stability of HLA-A and -B mRNAs in two cell lines appear to have slightly different turnover rates in three separate experiments. This finding suggests that varying stability for HLA-A and -B mRNAs could play some role in determining quantitative differential expression for certain HLA-A and -B antigens. The molecular basis for the observed different turnover rates is not known and remains to be investigated.

Because the steady state mRNA levels are determined by both mRNA degradation and production, and, in most cases, different HLA-A and -B mRNAs in cell lines studied have similar turnover rates, it is likely that differential production of HLA-A and -B mRNAs could be a primary determining factor for regulating differential expression of different HLA-A and -B antigens. We then studied how gene transcription contributes to the regulation of quantitative differential expression of HLA-A and -B genes. The initial nuclear run-on study showed that the newly synthesized HLA transcripts contribute only approximately 20% of the total prespliced HLA transcripts in nuclei (Figure 12). Due to the low quantity of newly synthesized HLA transcripts, we were unable to reliably determine the relative rate of transcription for different HLA-A and -B genes in cells. This finding also indicates that the processing of HLA pre-mRNAs is a critical rate-limiting step in the production of mature HLA mRNAs.

We then directed our effort to investigate whether differential splicing plays an important role in determining differential production of mature HLA-A and -B mRNAs. For this study, we used RT-PCR/DGGE and phosphor imaging to measure the relative quantities of HLA-A and -B transcripts before and after splicing of intron 2. The measurements were compared with those of mature cytoplasmic HLA mRNAs. The results suggested that nuclear HLA transcripts containing intron 2 can be proportionally or

differentially spliced, depending on the HLA alleles. Because it is more difficult to consistently generate sufficient quantities of first-strand HLA cDNA containing more introns and the amplicon of exon 2 of HLA gene is crucial for quantitation by DGGE, we limited our study of prespliced mRNA transcripts to those containing intron 2. The results of this study showed that the relative quantities of spliced mRNAs for various HLA-A or -B genes in nuclei and cytoplasm are about the same for all cell lines included in our study. In contrast, the relative quantities of HLA transcripts containing intron 2 for various HLA-A and -B genes are quite different from those of the spliced HLA-A and -B transcripts in nuclei and cytoplasm of some cell lines (Figure 15). Thus the results indicated that differential splicing of HLA transcripts plays a major role in determining differential quantitative expression of HLA-A and -B genes in cells.

Interestingly, in those cell lines showing differential splicing of HLA-A and -B pre-mRNAs, the relative quantities of unspliced HLA-A pre-mRNAs in nuclei of all these cells are higher than those of unspliced HLA-B pre-mRNAs, although the relative quantities of mature HLA-A mRNAs in most of these cell lines are lower than that of HLA-B mRNAs. This finding further supports the importance of differential splicing in regulating quantitative expression of HLA-A and -B genes. However, in cell lines showing proportional splicing of HLA-A and -B pre-mRNAs, the relative quantities of unspliced HLA-A pre-mRNAs and mature HLA-A mRNAs are higher than those of unspliced HLA-B pre-mRNAs and mature HLA-B mRNAs, respectively. These observations coincide with an earlier report that the basal level transcription of the HLA-A gene tends to be more efficient than that of the HLA-B gene due to the existence of a second NF-κB binding motif in the promoter of HLA-A gene (Girdlestone et al. 1993). These results also suggest that transcription of HLA gene is another major factor determining the quantitative differential expression of HLA-A and -B antigens. The mechanisms underlying the differential transcription and/or splicing and their contribution to the quantitative differential expression of HLA-A and -B antigens remain to be further defined.

CHAPTER 4
IN VITRO TRANSLATION STUDY OF HLA-A24 AND -B60 MRNAS

Introduction

As discussed in Chapter 3, quantitative differential expression of HLA-A and -B antigens is regulated by a combination of different steps that include gene transcription, pre-mRNA splicing, mRNA degradation, and translation. For mRNA translation, it appears that mRNAs for HLA-A24 and -B7 are more efficient in protein synthesis (Table 2). This finding suggests that translation of HLA mRNA could be an additional unique step in regulating expression of HLA antigen for certain specific alleles. Therefore, it is of interest to determine whether HLA-A24 mRNA is indeed more efficient in translation. For my study, I have focused on HLA-A24 protein synthesis because I have consistently found that HLA-A24 antigens are always more intensely expressed in all the studied HLA-A24 positive cell lines in spite of relatively low levels of mRNA.

Materials and Methods

Lymphoblastoid Cell Lines and RNA Preparation

EBV-transformed lymphoblastoid cell lines (LCLs) were selected from those described in Chapter 3. These cell lines were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% fetal calf serum, 1% antibiotic-antimycotic solution and 40 μg/ml gentamycin.

Rapid Amplification of HLA-A And -B cDNA Ends (RACE)

The 5' RACE is performed based on the protocol described by Frohman (1994) with some modification. Fifty micrograms of total cytoplasmic RNAs prepared from the selected cell lines were dephosphorylated with 3.5 units of calf intestinal phosphatase (CIP) (Boehringer Mannheim, Indianapolis, IN) in 50 µl of a buffer containing 50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5, 1 mM DTT, 1 unit/µl RNasin at 50°C for 1 hour. After digestion with 50 µg/ml of proteinase K at 37°C for 30 minutes, the mixture was extracted with phenol/chloroform and the RNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Thirty-eight micrograms of the dephosphorylated RNA was then decapped with 5 units of Tobacco acid pyrophosphatase (Epicentre, Madison, WI) in 50 µl of a buffer containing 50 mM sodium acetate, pH 6.0, 1 mM EDTA, 0.1% β-mercaptoethanol, 0.01% Triton X-100, 1 unit/µl of RNasin and 2 mM ATP at 37°C for 1 hour. The RNA was extracted with phenol/chloroform and precipitated with ethanol as described above. The decapped RNA was then ligated to an RNA oligonucleotide that was generated by in vitro transcription from plasmid pGbx-1 (kindly provided by Dr. Michael A. Frohman) and contains 132 nucleotides (Frohman, 1994). The ligation was carried out with 30 units of T4 RNA ligase (Epicentre, Madison, WI) in 30 µl of a mixture containing 33 mM Tris-HCl, pH 7.8, 66 mM potassium acetate, 10 mM $MgCl_2$, 0.5 mM DTT, 1 unit/µl RNasin, 0.1 mM ATP, 4 µg of RNA oligonucleotide and 10 µg of decapped RNA at 17°C for 16 hours. After extraction and precipitation, 6 µg of the ligation products were then used as templates for reverse transcription in 20 µl of a mixture containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM dNTPs, 0.01 M DTT, 0.5 unit of RNasin, 250 ng antisense-specific primer (5'-ACA GCT CCA(G) A(G)TG AC(T)C ACA-3') complementary to nucleotides 960-979 (in exon 5) of HLA-A and -B coding sequences and 200 units of MMLV reverse transcriptase at 37°C for 60 minutes, 42°C for 30 minutes and 50°C for 10 minutes. After inactivation of the reverse transcriptase, 5 µl of the RT mixture was directly used as template for PCR in 100 µl of a buffer containing 20

mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 10 μg/ml BSA, 0.2 mM dNTPs, 0.5 μM of each primer and 5 units of native *Pfu* DNA polymerase (Stratagene) for 35 cycles. Each cycle consisted of 94°C denaturation for 1 min, 60°C annealing for 1 sec and 72°C extension for 1 min. The sense primer sequence is 5'- CCA AGA CTC ACT GGG TAC TGC-3' and corresponds to nucleotides 62-82 of the RNA oligonucleotide. The antisense primer sequence is 5'-GCG ATG TAA TCC TTG CCG-3' and complementary to the coding sequence at nucleotides 429-446 of class I HLA mRNA. The PCR products containing 5' end sequences of HLA mRNA were directly cloned into a plasmid the pCR-Script Amp cloning vector (Stratagene, La Jolla, CA) according the manufacturer's protocol. Sequences of the cloned PCR products were determined by automated DNA sequencing.

The 3' RACE is also performed based on the protocol described by Frohman (1994). Briefly, 5 μg of total cytoplasmic RNA prepared from the 9075 or DC cell line was reverse transcribed in 30 μl of a buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM dNTPs, 0.01 M DTT, 0.5 unit of RNasin, 2 μg anchor primer (5'-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TTT T-3') and 200 units of M-MLV reverse transcriptase at 37°C for 60 minutes, 42°C for 30 minutes and 50°C for 10 minutes. After inactivation of the reverse transcriptase, 5 μl of the RT mixture was directly used as template for polymerase chain reaction (PCR) in 100 μl of a buffer containing containing 20 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 10 μg/ml BSA, 0.2 mM dNTPs, 0.5 μM of each primer and 5 units of native *Pfu* DNA polymerase (Stratagene, La Jolla, CA) for 35 cycles. Each cycle consisted of 94°C denaturation for 1 min, 60°C annealing for 1 sec and 72°C extension for 4 min. The PCR primer sequences are 5'-CGC CGT GGA TAG AGC AGG-3' (sense) and 5'-CCA GTG AGC AGA GTG ACG-3' (antisense). The sense primer corresponds to the coding sequence 218-235 of class I HLA mRNA, and the antisense primer corresponds to the 5' end of anchor primer. The PCR

products were then used as template for the nested PCR in which each cycle consisted of 94°C denaturation for 1 min, 60°C annealing for 1 sec and 72°C extension for 2 min. The primer sequences for the nested PCR are 5'- GCT GGC CTG GTT CTC CTT GG-3' (sense, corresponding to nucleotides 937-956 of HLA-A24 coding sequence) or 5'- GCT GTG GTG GTG CCT TCT GG-3' (sense, corresponding to nucleotides 808-827 of HLA-B60 coding sequence), and 5'- GAG GAC TCG AGC TCA AGC-3' (antisense, corresponding to a sequence in anchor primer). The products of the nested PCR were directly cloned into the pCR-Script Amp cloning vector (Stratagene, La Jolla, CA) according the manufacturer's protocol. The 3'-end sequence was determined by automated DNA sequencing.

Cloning of Full-length HLA-A24 and -B60 cDNAs By PCR

For cloning the whole length HLA cDNA, a PCR technique based on splicing by overlap extension (SOE) (Horton et al., 1989) was used. The 5' fragment of HLA cDNA was amplified from plasmid by using PCR in which a T7 promoter was incorporated into the 5' end for use in the subsequent synthesis of HLA transcripts. The 3' end sequence and a coding sequence were prepared also amplified from plasmids by using PCR, and the PCR products of these two fragments were mixed and used as templates for SOE PCR in which these two fragments with overlap sequences were jointed together to form the 3' fragment. *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used in PCR to reduce the possibility of misincorporation mutations in the PCR products. Standard PCR conditions were used for the amplification of template DNA fragments to be used in the subsequent SOE reactions (30 cycles of 1 min at 94°C, 30 sec at 60°C, and 2 min at 72°C following the final cycle, an additional 16-min incubation at 72°C). The PCR products corresponding to the 3' end HLA cDNA fragments and overlapping with the 5' end HLA cDNA fragments were purified by preparative agarose gel electrophoresis. The full-length HLA cDNAs were generated by another round of SOE PCR in which the 5' fragment and 3' fragment

were mixed and used as templates. The final PCR products contained a T7 promoter, the whole length HLA cDNA sequence and a poly(A) tail followed by a *Hin*d III restriction site and an anchor sequence. The anchor sequence was removed by digestion of the PCR products with *Hin*d III (Boehringer Mannheim, Indianapolis, IN). The whole length HLA heavy chain cDNA was then generated. The different fragments of HLA-cDNA used for SOE-PCR are shown in Figure 17.

Synthesis of Capped HLA-A24 and -B60 mRNAs by In Vitro Transcription

Five hundred nanograms of whole length HLA cDNA fragments generated as described above were used as templates for in vitro transcription in a volume of 20 µl at 37°C for 3 hours using T7 RNA polymerase according to the manufacturer's protocol (mMachinemMessage^{TM} In Vitro Transcription Kits) (Ambion Inc., Austin, TX) to synthesize capped HLA mRNA. After 3 hours incubation, 7.5 units RNase-free DNase I was added and incubated at 37°C for 60 minutes to degrade the template DNA. The in vitro synthesized RNA transcripts were recovered with LiCl precipitation and further cleaned using an RNeasy spin column (QIAGEN Inc., Chatsworth, CA). The concentrations of the synthesized RNA were measured by absorbance at 260 nm.

Synthesis of HLA-A24 and -B60 Proteins by In Vitro Translation

After heating to 67°C for 10 min, 25 ng/µl of HLA-A or -B mRNAs transcribed in vitro were translated at 30°C for 2 hours in a 25 µl reaction mixture containing 17.5 µl of nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI), 20 units of RNasin, 20 µM amino acids minus methionine, and 20 µCi [^{35}S]methionine (Amersham Life Science, Inc., Arlington Heights, IL). Five microliters of each translation reaction mixture were analyzed by conventional SDS-PAGE (10% acrylamide) followed by fixation and autoradiography or phosphor imaging of the dried gel. The translation products were also analyzed by SDS-PAGE followed by immunoblotting with 171.4 anti-HLA-A and -B

heavy chain (Hc) monoclonal antibody (mAb) (Kao et al., 1990) and autoradiography to confirm that they are HLA heavy chains.

Autoradiography and Phosphor Imaging

For autoradiography, the SDS-PAGE gels were exposed to Kodak Biomax MR film (Kodak Scientific Imaging Systems, Rochester, NY) with an intensifying screen for 24 hr at -70°C. For phosphor imaging, the gels were exposed to a Phosphorscreen (Molecular Dynamics, Inc., Sunnyvale, CA) for 48 hours at room temperature. The radioactivity of each specific protein band was quantified using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Cloning of HLA-A24 and -B60 cDNA Ends by RACE PCR

Although coding sequences for many class I HLA genes have been documented (Mason and Parham 1998), the non-coding sequences at 5' end or 3' end of class I HLA mRNAs are available only for a few HLA genes, not including HLA-A24 or -B60 (Srivastava et al., 1985). In order to obtain the full-length HLA-A24 and -B60 cDNAs, we first obtained the 5' and 3' end sequences of HLA-A24 and -B60 cDNAs by RACE PCR. The RACE protocol we used allowed us to amplify only the 5' end with intact 5'-UTR (Frohman 1994). The cloned 5'-UTR sequences of HLA-A24 and -B60 are shown in Table 3. After comparing the 5'-UTRs of the cloned HLA-A24 and -B60 mRNAs, we found two types of 5'-UTRs for HLA-A24 mRNAs. One has 40 nucleotides and the other has 22 nucleotides (Table 3). The cloned 5'-UTR sequence of HLA-B60 only consists of 21 nucleotides. So far, the long 5'-UTR was not found in 5'-end clones of HLA-A2,-B7 and -B35 mRNAs and may be unique for HLA-A24. After comparing these sequences with the published 5'-UTR sequences of HLA-A and -B mRNAs, it was found that the

Table 3 5' end sequences of HLA-A24 and -B60 mRNAs

HLA	5' end sequence*
A24 (long)	5'-ACGCACCCACCGGGACUCAGAUUCUCCCCAGACGCCGAGGAUGGCCGUCAUGGCG-3'
A24	5'-AGAUUCUCCCCAGACGCCGAGGAUGGCCGUCAUGGCG-3'
B60	5'-AGAAUCUCCUCAGACGCCGAG AUGCGGGUCACGGCA-3'

*Coding sequence is underlined.

short 5'-UTRs of HLA-A24 and -B60 mRNAs are highly similar to those of other HLA-A and -B mRNAs.

The 3'-UTR sequences of HLA-A24 and -B60 mRNAs were also obtained (Figure 16) There is about 15% difference between the 3'-UTR sequence of HLA-A24 mRNA and that of HLA-B60 mRNA throughout the 3' trail of about 430-nucleotides.

Cloning of Full-length HLA-A24 and -B60 heavy chain cDNAs by PCR

Because we were unsuccessful in using PCR to directly generate the full-length cDNA, the full-length HLA cDNAs were constructed using a PCR technique based on SOE (Horton et al., 1989). We first cloned part of HLA coding sequences, the 5' end sequences and the 3' end sequences of HLA-A24 (Figure 17A) and -B60 (Figure 17B) into plasmids. The HLA cDNA fragments with overlap sequences were then amplified from the plasmids and spliced together by using SOE PCR (Figure 17). A T7 promoter was incorporated into the 5' end of each HLA cDNA. Poly$(A)_{17}$ at the 3' end was followed by a *Hin*d III restriction site and an anchor sequence. After digestion of the final PCR products with *Hin*d III, the whole length HLA-A 24 and -B60 heavy chain cDNAs were used as templates for in vitro transcription to synthesize capped HLA mRNAs for translation study.

```
>A24        AAAGUGUGAG ACAGCUGCCU UGUGUGGGAC UGAGAGGCAA GAGUUGUUC:
>B60          --CC---- -------U-- ----A----- -----U---G --U--C---A

>A24        CUGCCCUUCC CUUUGUGACU UGAAGAACC: CUGACUU:UG UUUCUGCAAA
>B60        --:--:-C-- ---------- -C----G--U ---G-A-C-C ----------

>A24        GGCACCUGCA UGUGUCUGUG UUCAUGUAGG CAUAAUGUGA GGAGGUGGGG
>B60        --------A- --------C- -C-C---UA- -C-------- --------A-

>A24        AGACCACCCC ACCCCCAUGU CCACCAUGAC CC:UCUUCCC ACGCUGACCU
>B60        ----:-G--- ------G--- ----UG---- --C-G----- -U--------

>A24        GUGCUCCCUC CCCAAUCAUC UUUCCUGUUG CAGAGAGGUG GGGCUGAGGU
>B60        ---U-U---- ----G----- ----U----C ---------- ------:-A-

>A24        GUCUCCAUCU CUGUCUCAAC UUCAUGGUGC ACUGAGCUGU AACUUCUUCC
>B60        ---------- ---------- --U---:--- ---------C --------A-

>A24        UUCCCUAUU: AAAAUUAGAA CCUGAGUAUA AAUUUACUUU CUCAAAUUCU
>B60        -------C-G -----A---- U----A---- -----GU--- -------AU-

>A24        UGCCAUGAGA GGUUGAUGAG UUAAUUAAAG GAGAAGAUUC CUAAAAUUUG
>B60        ---U------ --------GA ---------U A--UCA---- --GG------

>A24        AGAGACAAAA UAAAUGGAAC ACAUGAGAAC CUUC
>B60        -A---GC--- ----:-:-:- :-:------- ----
```

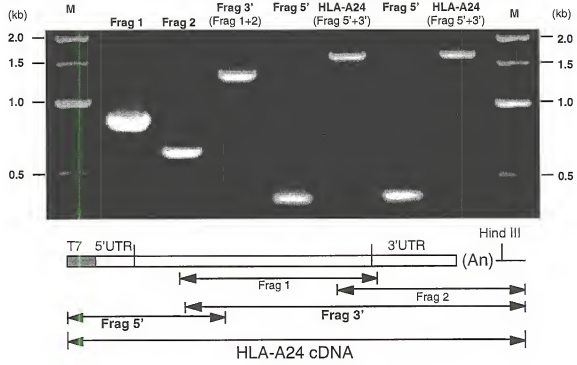
Figure 16 The 3'-UTR sequences for HLA-A24 and -B60 mRNAs. ":" denotes a deletion introduced to maximize the homology.

In Vitro Translation study of HLA-A24 and -B60 mRNAs

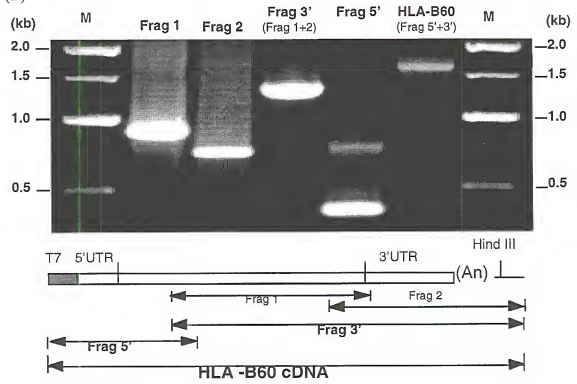
After digestion with *Hin*d III, the HLA-A24 and -B60 cDNA constructs were used to prepare capped HLA mRNA by in vitro transcription. The same amounts of capped HLA-A24 and -B60 mRNAs (25 ng/μl) were used for sythesizing HLA-A24 and -B60 proteins by in vitro translation in the rabbit reticulocyte system. The same amounts of translation mixtures were analyzed by SDS-PAGE, phosphor imaging and immunoblotting. The results shown in Figure 18 indicate that more HLA-A24 heavy chains were synthesized from HLA-A24 mRNAs than HLA-B60 heavy chains from HLA B60 mRNAs. In addition, the long HLA-A24 mRNA is shown to be more efficient than

Figure 17 Cloning of HLA-A24 and -B60 heavy chain cDNAs by PCR. (A) PCR fragments of HLA-A24 cDNAs. One is with short UTR (HLA-A24), and the other is with longer UTR (HLA-A24L). Fragment 1 (Frag 1) corresponds to a HLA mRNA sequence from +251 to +1122 (A in first ATG initiation codon is designated as +1.). Fragment 2 (Frag 2) corresponds to the sequence from +937 to the anchor sequence. 5' fragment (Frag 5') represents a T7 promoter plus the sequence from -22 (for HLA-A24) or -40 (for HLA-A24L) to +340. (B) PCR fragments of HLA-B60 cDNAs. Fragment 1 (Frag 1) correponds to a HLA mRNA sequence from +218 to +1122 (A in first ATG initiation codon is designated as +1.). Fragment 2 (Frag 2) corresponds to the sequence from +808 to the anchor sequence. 5' fragment (Frag 5') represents a T7 promoter plus the sequence from -22 to +340. M: DNA markers.

(A)


(kb)
M
Frag 1
Frag 2
Frag 3'
(Frag 1+2)
Frag 5'
HLA-A24
(Frag 5'+3')
Frag 5'
HLA-A24
(Frag 5'+3')
M
(kb)
2.0
1.5
1.0
0.5
Hind III
T7
5'UTR
3'UTR
(An)
Frag 1
Frag 2
Frag 5'
Frag 3'
HLA-A24 cDNA

(B)


(kb)
M
Frag 1
Frag 2
Frag 3'
(Frag 1+2)
Frag 5'
HLA-B60
(Frag 5'+3')
M
(kb)
2.0
1.5
1.0
0.5
Hind III
T7
5'UTR
3'UTR
(An)
Frag 1
Frag 2
Frag 3'
Frag 5'
HLA -B60 cDNA

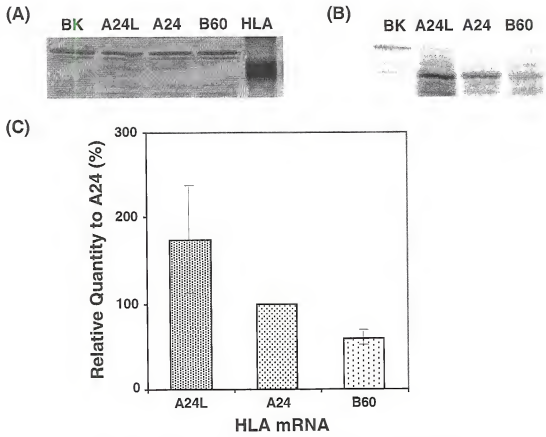


Figure 18 In vitro translation study of HLA-A24 and HLA-B60 mRNAs.
(A) Representative immunoblot of a SDS-PAGE gel for newly synthesized heavy chains of HLA-A24 and -B60. The [^{35}S]methionine-labeled HLA-A24 and -B60 proteins generated by in vitro translation of long HLA-A24 (A24L), short HLA-A24 (A24) and HLA-B60 (B60) mRNAs were analyzed by SDS-PAGE and immunoblotting. The protein bands on the blot membrane was quantified by phosphor imaging. BK: control reaction mixture without addition of HLA mRNAs. HLA: purified HLA heavy chain. Two bands with 44 kD and 40 kD were shown in the purified HLA heavy chain. The 40 kD represent the degraded HLA. (B) Phosphor image of the immunoblot in (A). (C) The relative quantities of HLA proteins synthesized by in vitro translation of HLA-A24L, HLA-A24 and HLA-B60 mRNAs:
Relative quantity of HLA-A24 proteins synthesized by short HLA-A24 mRNAs is designated as 100%;
Relative quantity of HLA-A24 proteins synthesized by long HLA-A24 mRNAs = [(phosphor density of A24L)/(phosphor density of A24)] x 100%;
Relative quantities of HLA-B60 proteins synthesized HLA-B60 mRNAs = [(phosphor density of B60 x 9)/(phosphor density of A24 x 5)] x 100%.
Each value represents the mean of four separate measurements. Because HLA-B60 protein has fewer methionine residues (5/molecule) than HLA-A24 protein (9/molecule), the quantity of HLA-B60 protein determined by phosphor imaging was calibrated based on its number of methionine residues.

the short HLA-A24 mRNA in synthesizing HLA-A24 heavy chains. HLA heavy chains were identified based on molecular weight and immunoblotting with 171.4 anti-HLA-Hc mAb (Kao et al., 1990) (Figure 18). The amounts of HLA heavy chains produced by different mRNAs in four separate experiments were quantified and normalized according to their methionine contents against HLA heavy chains synthesized from the short HLA-A24 mRNAs. The results shown in Figure 18C indicated that the efficiency of HLA heavy chain synthesis were 174%, 100% and 59% for long HLA-A24 mRNA, short HLA-A24 mRNA and HLA-B60 mRNA, respectively.

Discussion

The primary goal of this study was to determine whether HLA-A24 mRNA is more efficient in protein translation as suggested by our earlier quantitative correlation study between cytoplasmic HLA mRNA and HLA protein expression (Table 2 in Chapter 3). To accomplish this goal we studied the in vitro protein translation of HLA-A24 and -B60 mRNAs from the 9075 cell line. This cell line was chosen for our study because of the consistent reverse correlation observed between relative quantities of HLA-A24 and -B60 mRNAs and that of HLA-A24 and -B60 proteins.

Although the gene sequences of class I HLA have been documented (Mason and Parham 1998) and the regulatory elements of the promoter region have been identified (Cereb and Yang 1994), there are few reports on the initiation sites of the transcription of HLA genes. Also, the reported 5'-UTR sequences are often incomplete. In order to obtain the full-length HLA cDNA for our in vitro protein translation study, the sequence information of 5' UTR and 3'-UTR for HLA-A24 and -B60 mRNAs had to be obtained. By using the RACE technique, we were able to obtain 5'-UTR and 3'-UTR sequences and to clone the full-length HLA cDNAs. In the 5' RACE approach, we first dephosphorylated all the degraded mRNA with CIP to render them inert during the ensuing ligation reaction.

Then, the intact capped mRNAs were treated with tobacco acid pyrophosphatase. This treatment makes them active for the subsequent ligation with an RNA anchor oligonucleotide (Frohman, 1994). By using this approach, we identified two types of HLA-A24 mRNA with two different lengths of 5'-UTRs (Table 3). Although we did not find any HLA-B60 mRNA with long 5'-UTR, this possibility have not been excluded.

Results of the translation study in rabbit reticulocyte lysate system using HLA-A24 and -B60 mRNAs synthesized in vitro indeed demonstrated that HLA-A24 transcripts are more efficient than HLA-B60 mRNAs in synthesizing HLA proteins. The newly synthesized HLA heavy chains are identified based on molecular weight and immunoreactivity to an anti-HLA-heavy chain monoclonal antibody. Although there is some nonspecific binding of 171.4 mAb to other proteins present in rabbit reticulocyte lysate, the inclusion of a control mixture of rabbit reticulocyte lysate enabled us to identify the newly synthesized HLA-heavy chains. We did not observe any nonspecific binding of 171.4 to molecular weight standards. The reasons for the observed high background on our immunoblot are not clear. To obtain more accurate quantitative results, the newly synthesized HLA heavy chains were measured by phosphor imaging (Figure 18B). The results shown in Figure 18C indicated that long HLA-A24 transcript is more efficient than the short HLA-A24 transcript and that the short HLA-A24 transcripts are about 2 times more efficient than HLA-B60 transcripts in making HLA heavy chains. Based on our previous measurements of the relative quantities of HLA-A24 and -B60 mRNAs (41% vs. 59%) in the 9075 cell line, and the protein translation efficiency determined by the present study, we predict that relative quantities of HLA-A24 and -B60 proteins in the 9075 cell line will be 54% vs. 46%. This calculation, however, did not take into consideration of a small percentage of HLA-A24 transcripts that are present in long form, which are about 3-4 times more efficient than HLA-B60 transcripts in protein translation. Thus, the calculated values are close to the relative quantities of HLA-A24 and -B60 proteins (63% vs. 37%) observed in the 9075 cell line.

Although our results showed that HLA-A24 transcripts are more efficient in protein translation in the rabbit reticulocyte lysate system, the exact mechanism for this finding is not clear. When the 5' end sequences of HLA-A24 and -B60 mRNAs are compared, the following features are noticed: (1) There are two AUG codons, separated by 6 nucleotide residues at the beginning of coding sequence for HLA-A24 mRNA (Table 1). The translation may be initiated at either of these two codons (Srivastava et al., 1985), whereas only the first AUG is found at the beginning of HLA-B60 mRNA coding sequence. (2) The 5'-UTR of HLA-B60 mRNA has a one-nucleotide deletion immediately before the first AUG codon comparing to that of HLA-A24 mRNA. (3) No exact Kozak sequence GCCA(G)CC**AUG**G (Kozak, 1984; Kozak, 1986; Kozak, 1987) is found in the 5'-UTR of either HLA-A24 or HLA-B60. However, the sequence proximal to the second AUG codon of HLA-A24 mRNA (GCCGTC**AUG**G) is more similar to the Kozak sequence than the sequence proximal to the first AUG codon of HLA-B60 codon (GCCGAGA**UG**C). It is likely that these differences may contribute to the enhanced protein translation efficiency by HLA-A24 mRNA. Moreover, there are about 10% and 15% difference between HLA-A and -B in coding sequences and 3'-UTR, respectively. Because the 3'-UTR could also play some role in regulating mRNA translation (Jacobson and Peltz, 1996), the possible effect of 3'-UTR on the observed differential translation of HLA-A24 and -B60 mRNAs could not be excluded.

In this study, we also found that the long HLA-A24 mRNA is more efficient for protein translation than the short form. This finding indicated that the long 5'-UTR may enhance the protein translation initiation. The mechanism for the enhanced translation of long HLA-A24 mRNA is not clear. Because no secondary structures are found within this long 5'-UTR, it is likely that the longer 5'-UTR can accumulate extra 40S ribosomal subunits, which may account for its translational advantage (Kozak, 1991).

Because the rabbit reticulocyte lysate system had been shown to be efficient for in vitro protein translation and was commercially available (Pelham and Jackson, 1976;

Shields and Blobel, 1978), this system was chosen for our study. However, the rabbit reticulocyte lysate is a heterologous system, and the results obtained from this system may not represent the actual situation in human lymphoblastoid cells. At present, the exact mechanism responsible for the enhanced HLA-A24 protein translation has yet to be further elucidated. Nevertheless, the results of this study suggested that different protein translation rates could contribute to genetically predetermined differential quantitative expression of HLA-A and -B antigens.

CHAPTER 5
SUMMARY AND FUTURE DIRECTION

Earlier studies have shown that different specific HLA-A and -B antigens are differentially expressed in cells. Their relative quantities are genetically predetermined and inherited according to Mendelian law (Kao and Riley, 1993). In order to determine the regulatory mechanisms underlying the observed phenomenon, we first studied the turnover of HLA proteins in lymphoblastoid cell lines and found that different HLA-A and -B antigens are proportionally degraded. When the relative quantities of HLA proteins were correlated with those of HLA mRNAs, it was found that, in most of the studied cell lines, the relative quantities of different HLA-A and -B proteins are proportional to those of their respective mRNAs.

In addition, different HLA-A and -B proteins have similar stabilities (Figure 8) and the levels of different HLA-A and -B proteins are proportional to their mRNA levels in most lymphoblastoid cell lines. These findings indicate that the availability of functional HLA mRNAs determines the differential quantitative expression of HLA antigens. Because the steady-state levels of HLA mRNAs are regulated by mRNA production and degradation, the involvement of both steps in regulating the differential quantitative expression of HLA antigens was studied. First, we measured the relative quantities of different HLA-A and -B mRNAs before and after the cells were treated with DRB, an inhibitor of RNA polymerase II. The results of this study showed that different HLA-A and -B mRNAs were proportionally degraded in five out of seven cell lines studied, and that the stabilities of HLA-A and -B mRNAs in the remaining two cell lines appear to have slightly different turnover rates. This finding suggests that the varying stability for HLA-A

and -B mRNAs only plays a minor role in determining differential quantitative expression for certain HLA-A and -B antigens.

Next, we studied the role of mRNA production. The results of our PCR-based nuclear run-on study showed that newly synthesized HLA transcripts only account for less than 20% of total HLA pre-mRNAs. The presence of relatively large amount of pre-mRNA suggests that pre-mRNA splicing could be a rate-limiting step in regulating HLA mRNA production. This finding also prevents us from accurately measuring the newly synthesized HLA transcripts by using a PCR-based nuclear run-on method. We therefore performed experiments to determine the relative quantities of unspliced HLA-A and -B transcripts and those of spliced HLA-A and -B transcripts. It was found that different HLA-A and -B pre-mRNAs in nuclei are not proportional to their mature cytoplasmic mRNAs in five of seven HLA-phenotyped lymphoblastoid cell lines. The differences are quite significant for some of the cell lines. These results suggest that the splicing of pre-mRNA and gene transcription are critical in regulating the genetically predetermined differential expression of HLA-A and -B antigens in different cell lines.

Although the relative quantities of different HLA-A and -B antigens are proportional to the relative amounts of their respective mRNAs in most lumphoblastoid cell lines, in cell lines positive for the HLA-A24 or -B7, the HLA-A24 and -B7 proteins appear to be overexpressed. This observation suggests that mRNAs for certain HLA antigens may be more efficient in synthesizing HLA heavy chains. We therefore selected the 9075 cell line, which is positive for HLA-A24 and -B60, to study whether translation of mRNA plays a role in influencing the differential quantitative expression of HLA-A and -B antigens. In vitro translation studies indicated that HLA-A24 and -B60 mRNAs synthesized in vitro indeed have different translation rates. Our results showed that HLA-A24 mRNA is more efficient than HLA-B60 mRNA in synthesizing HLA proteins. This observation supported the hypothesis that differential mRNA translation could play a role in determining the differential quantitative expression of HLA antigens for certain HLA phenotypes.

In summary (Table 4), the results of my study indicate that the quantitative differential expression of HLA-A and -B antigens is determined by combinations of multiple steps. These steps include gene transcription, pre-mRNA splicing, mRNA degradation, and/or mRNA translation depending on specific HLA alleles in different individuals. Among them, gene transcription and pre-mRNA splicing play the most prominent roles. For certain specific HLA antigens, i.e. HLA-A24, protein translation also plays a significant role.

Table 4 Contribution of different controlling steps to the regulation of differential quantitative expression of different HLA-A and -B antigens in the studied LCLs.

LCL	HLA-A&-B Phenotypes	Gene transcription*	Pre-mRNA splicing	mRNA turnover	mRNA translation	protein turnover
9005	A3, B27	+?	+	-	-	ND
9027	A29, B44	+	-	+	-	-
9067	A2, B27	+	-	+	-	-
9068	A2, B35	+?	ND	-	-	-
SH	A2, A3, B7, B44,	+?	ND	ND	+	ND
CG	A2-var, A3, B7, B45	+?	ND	ND	+	ND
9075	A24, B60	+?	+	-	+	ND
DC	A11, A24, B35, B60	+?	+	-	+	ND
9001	A24, B7	+?	+	-	+	-
9028	A24, B60, B61	+?	+	-	+	-

+: plays a role in determining the differential quantitative expression of HLA-A and -B antigens in this LCL.
-: does not play a role in determining the differential quantitative expression of HLA-A and -B antigens in this LCL.
*: Conclusion for this step is inferred from studies of other steps.
ND: not determined.

The differential quantitative expression of class I HLA antigens could also be regulated by other steps, including the association of HLA heavy chain with β2m or chaperones, the availability of antigenic peptides, and the transportation of the assembled antigens to the cell surface. Although all of these additional potential regulatory steps have not been studied, they are not likely to play significant roles in regulating the observed differential quantitative expression. If these additional steps were important in regulating the differential expression of HLA antigens, we would not have observed the proportional correlation between the relative amounts of HLA-A and -B proteins and those of their respective HLA-A and -B mRNAs in most cell lines (Table 2).

Although the research works presented in this dissertation have identified the critical steps for regulating differential quantitative expression of HLA antigens, the exact molecular mechanisms directly responsible for differential gene transcription, differential pre-mRNA splicing, and differential protein translation remain to be elucidated. The sequence differences among different HLA-A and -B genes scattered in both coding and non-coding regions could be involved in regulating the genetically predetermined differential quantitative expression of HLA-A and -B antigens. Therefore, it would be of interest to determine how 5'-UTR, 3'-UTR and/or coding sequences determine the observed different efficiencies in protein translation by different HLA mRNAs. This study can be conducted by constructing different HLA-A24-B60 cDNA hybrids and performing the in vitro translation study. By switching the 5'-UTR of HLA-A24 mRNA to that of HLA-B60 mRNA, for example, we will be able to learn whether the sequence difference in their 5'-UTRs plays a certain role in regulating the observed differential translation. To investigate how HLA-A and -B pre-mRNAs are spliced differentially, northern blot of the nuclear RNAs with probes derived from different introns could be used to determine whether introns of different HLA pre-mRNAs are spliced following the same order or not. It is also important to identify the specific sequence(s) responsible for the observed differential splicing of HLA-A and -B pre-mRNAs. Further comparative study on genomic

structures of HLA-A and -B genes from unrelated individuals will enable us to answer the questions regarding the underlying mechanisms for differential gene transcription. A deeper understanding of these different regulatory steps will allow us to further elucidate the biological and genetical importance of differential quantitative expression of HLA antigens in determining varying disease susceptibilities such as immune surveillance of tumor cells and recovery from virus infection in the future.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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